Metabolically engineered cells for the production of resveratrol or an oligomeric or glycosidically-bound derivative thereof

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(54) Title: METABOLICALLY ENGINEERED CELLS FOR THE PRODUCTION OF RESVERATROL OR AN OLIGOMERIC OR GLYCOSIDICALLY-BOUND DERIVATIVE THEREOF

(57) Abstract: A recombinant micro-organism producing resveratrol by a pathway in which phenylalanine ammonia lyase (PAL) produces trans-cinnamic acid from phenylalanine, cinnamate 4- hydroxylase (C4H) produces 4-coumaric acid from said trans- cinnamic acid, 4-coumarate-CoA ligase (4CL) produces 4- coumaroyl CoA from said 4-coumaric acid, and resveratrol synthase (VST) produces said resveratrol from said 4- coumaroyl CoA, or in which L-phenylalanine- or tyrosine- ammonia lyase (PAL/TAL) produces 4-coumaric acid, 4- coumarate-CoA ligase (4CL) produces 4-coumaroyl CoA from said 4-coumaric acid, and resveratrol synthase (VST) produces said resveratrol from said 4-coumaroyl CoA. The micro-organism may be a yeast, fungus or bacterium including Saccharomyces cerevisiae, E. coli, Lactococcus lactis, Aspergillus niger, or Aspergillus oryzae.

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METABOLICALLY ENGINEERED CELLS FOR THE PRODUCTION OF RESVERATROL OR AN OLIGOMERIC OR GLYCOSIDICALLY-BOUND DERIVATIVE THEREOF.

FIELD OF THE INVENTION

This invention relates generally to the production of the polyphenol resveratrol or an oligomeric or glycosidically bound derivative thereof such as its β-glucoside piceid using microbial cells. Furthermore, it relates to the use of naturally occurring or recombinant micro-organisms that produce resveratrol or such a derivative for production of food, feed and beverages.

BACKGROUND OF THE INVENTION

Production of chemicals from micro-organisms has been an important application of biotechnology. Typically, the steps in developing such a bio-production method may include 1) selection of a proper micro-organism host, 2) elimination of metabolic pathways leading to by-products, 3) deregulation of desired pathways at both enzyme activity level and the transcriptional level, and 4) overexpression of appropriate enzymes in the desired pathways. In preferred aspect, the present invention has employed combinations of the steps above to redirect carbon flow from phenylalanine or tyrosine through enzymes of the plant phenylpropanoid pathway which supplies the necessary precursor for the desired biosynthesis of resveratrol.

Resveratrol (or 3,4,5-trihydroxystilbene) is a phytophenol belonging to the group of stilbene phytoalexins, which are low-molecular-mass secondary metabolites that constitute the active defence mechanism in plants in response to infections or other stress-related events. Stilbene phytoalexins contain the stilbene skeleton (*trans*-1,2-diphenylethylene) as their common basic structure: that may be supplemented by addition of other groups as well (Hart and Shrimpton, 1979, Hart, 1981). Stilbenes have been found in certain trees (angio-sperms, gymnosperms), but also in some herbaceous plants (in species of the *Myrtaceae*, *Vitaceae* and *Leguminosae* families). Said compounds are toxic to pests, especially to fungi, bacteria and insects. Only few plants have the ability to synthesize stilbenes, or to produce them in an amount that provides them sufficient resistance to pests.

The synthesis of the basic stilbene skeleton is pursued by stilbene synthases. So far, two enzymes have been designated as a stilbene synthase; pinosylvine synthase and resveratrol synthase. To date, the groundnut (*Arachis hypogaea*) resveratrol synthase has been characterised in most detail, such that most of the properties are known (Schoppner and Kindl, 1984). Substrates that are used by stilbene synthases are malonyl-CoA, cinnamoyl-CoA or coumaroyl-CoA. These substances occur in every plant because they are used in the biosynthesis of other important plant constituents as well such as flavonoids, flower pigments and lipids.

Resveratrol (Fig. 1 *trans*-form) consists of two closely connected phenol rings and belongs therefore to the polyphenols. While present in other plants, such as eucalyptus, spruce, and lily, and in other foods such as

mulberries and peanuts, resveratrol's most abundant natural sources are *Vitis vinifera*, *-labrusca*, and *-muscadine (rotundifolia)* grapes, which are used to make wines. The compound occurs in the vines, roots, seeds, and stalks, but its highest concentration is in the skin (Celotti *et al.*, 1996), which contains 50-100 µg/g. (Jang *et al.* 1997). During red wine vinification the grape skins are included in the must, in contrast to white wine vinification, and therefore resveratrol is found in small quantities in red wine only. Resveratrol has, besides its antifungal properties, been recognized for its cardioprotective- and cancer chemopreventive activities; it acts as a phytoestrogen, an inhibitor of platelet aggregation (Kopp *et al*, 1998; Gehm *et al* 1997; Lobo *et al* 1995), and an antioxidant (Jang *et al.*, 1997; Huang 1997). These properties explain the so-called French Paradox, i.e. the wine-drinking French have a low incidence of coronary heart disease despite a low-exercise, high-fat diet. Recently it has been shown that resveratrol can also activate the SIR2 gene in yeast and the analogous human gene SIRT1, which both play a key role in extending life span. Ever since, attention is very much focused on the life-span extending properties of resveratrol (Hall, 2003, Couzin, 2004).

American health associations, such as the Life Extension Foundation, are promoting the vast beneficial effects of this drug, and thereby propelling the ideal conditions for a successful commercialisation. Present production processes rely mostly upon extraction of resveratrol, either from the skin of grape berries, or from Knot weed. This is a labour intensive process and generates low yield which, therefore, prompts an incentive for the development of novel, more efficient and high-yielding production processes.

In plants, the phenylpropanoid pathway is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignins, salicylates, coumarins, hydroxycinnamic amides, pigments, flavonoids and phytoalexins. Indeed formation of resveratrol in plants proceeds through the phenylpropanoid pathway. The amino acid L-phenylalanine is converted into *trans*-cinnamic acid through the non-oxidative deamination by L-phenylalanine ammonia lyase (PAL) (Fig 2). Next, *trans*-cinnamic acid is hydroxylated at the *para*-position to 4-coumaric acid (4-hydroxycinnamic acid) by cinnamate-4-hydroxylase (C4H), a cytochrome P450 monooxygenase enzyme, in conjunction with NADPH:cytochrome P450 reductase (CPR). The 4-coumaric acid, is subsequently activated to 4-coumaroyl-CoA by the action of 4-coumarate-CoA ligase (4CL). Finally, resveratrol synthase (VST) catalyses the condensation of a phenylpropane unit of 4-coumaroyl-CoA with malonyl CoA, resulting in formation of resveratrol.

Recently, a yeast was disclosed that could produce resveratrol from 4-coumaric acid that is found in small quantities in grape must (Becker *et al.* 2003). The production of 4-coumaroyl-CoA, and concomitant resveratrol, in laboratory strains of *S. cerevisiae*, was achieved by co-expressing a heterologous coenzyme-A ligase gene, from hybrid poplar, together with the grapevine resveratrol synthase gene (*vst1*). The other substrate for resveratrol synthase, malonyl-CoA, is already endogenously produced in yeast and is involved in *de novo* fatty-acid biosynthesis. The study showed that cells of *S. cerevisiae* could produce minute amounts of resveratrol, either in the free form or in

the glucoside-bound form, when cultured in synthetic media that was supplemented with 4-coumaric acid.

However, said yeast would not be suitable for a commercial application because it suffers from low resveratrol yield, and requires addition of 4-coumaric acid, which is only present in few industrial media. In order to facilitate and broaden the application of resveratrol as both a pharmaceutical and neutraceutical, it is therefore highly desirable to obtain a yeast that can produce resveratrol directly from glucose, without addition of 4-coumaric acid.

A recent study (Ro and Douglas, 2004) describes the reconstitution of the entry point of the phenylpropanoid pathway in *S. cerevisiae* by introducing PAL, C4H and CPR from Poplar. The purpose was to evaluate whether multienzyme complexes (MECs) containing PAL and C4H are functionally important at this entry point into phenylpropanoid metabolism. By feeding the recombinant yeast with [3H]-phenylalanine it was found that the majority of metabolized [3H]-phenylalanine was incorporated into 4-[3H]-coumaric acid, and that phenylalanine metabolism was highly reduced by inhibiting C4H activity. Moreover, PAL-alone expressers metabolized very little phenylalanine into cinnamic acid. When feeding [3H]-phenylalanine and [14C]-*trans*-cinnamic acid simultaneously to the triple expressers, no evidence was found for channeling of the endogenously synthesized [3H]-*trans*-cinnamic acid into 4-coumaric acid. Therefore, efficient carbon flux from phenylalanine to 4-coumaric acid via reactions catalyzed by PAL and C4H does not appear to require channeling through a MEC in yeast, and sheer biochemical coupling of PAL and C4H seems to be sufficient to drive carbon flux into the phenylpropanoid pathway. In

yet another study (Hwang *et al.*, 2003) production of plant-specific flavanones by *Escherichia coli* was achieved through expression of an artificial gene cluster that contained three genes of a phenyl propanoid pathway of various heterologous origins; PAL from the yeast *Rhodotorula rubra*, 4CL from the actinomycete *Streptomyces coelicolor*, and chalcone synthase (CHS) from the licorice plant *Glycyrrhiza echinata*. These pathways bypassed C4H, because the bacterial 4CL enzyme ligated coenzyme A to both *trans*-cinnamic acid and 4-coumaric acid. In addition, the PAL from *Rhodotorula rubra* uses both phenylalanine and tyrosine as the substrates. Therefore, *E. coli* cells containing the gene clusters and grown on glucose, produced small amounts of two flavanones, pinocembrin (0.29 g/l) from phenylalanine and naringenin (0.17 g/l) from tyrosine. In addition, large amounts of their precursors, 4-coumaric acid and *trans*-cinnamic acid (0.47 and 1.23 mg/liter respectively), were acumulated. Moreover, the yields of these compounds could be increased by addition of phenylalanine and tyrosine.

Whereas the enzyme from dicotylic plants utilizes only phenylalanine efficiently, several studies indicated that PAL from monocotylic plants, and some micro-organisms, utilizes tyrosine as well (Rösler *et al.*, 1997). In such reactions the enzyme activity is designated tyrosine ammonia lyase (TAL, figure 3). Conversion of tyrosine by TAL results in the direct formation of 4-coumaric acid without the intermediacy of C4H and CPR. Mostly both activities reside on the same polypeptide and have very similar catalytic efficiencies, in spite of large differences in Km and turnover number. However, most PAL/TAL enzymes from plants prefer phenylalanine rather than tyrosine. The level of TAL activity is mostly lower than PAL activity, but the

magnitude of this difference varies over a wide range. For example, the parsley enzyme has a Km for phenylalanine of 15-25 µM and for tyrosine 2.0-8.0 mM with turnover numbers 22 s^{-1} and 0.3 s^{-1} respectively. In contrast, the maize enzyme has a Km for phenylalanine only 15-fold higher than for tyrosine, and turnover numbers about 10-fold higher. Moreover, in the red yeasts, *Rhodotorula glutinis* (*Rhodosporidium toruloides*) and -*rubra*, the TAL catalytic activity is close to the PAL catalytic activity with a ratio of TAL/PAL of approximately 0.58. It is believed that the PAL enzyme in these yeasts degrades phenylalanine as a catabolic function and the *trans*-cinnamic acid formed is converted to benzoate and other cellular materials, whereas in plants it is thought to be merely a regulatory enzyme in the biosynthesis of lignin, isoflavonoids and other phenylpropanoids.

Recently, an open reading frame was found in the bacterium *Rhodobacter capsulatus* that encodes a hypothetical biosynthetic tyrosine ammonia lyase (TAL) that is involved in the biosynthesis of the chromophore of the photoactive yellow protein (Kyndt *et al.*, 2002). This was the first time that a PAL-homologous gene was found in bacteria. The TAL gene was isolated and overproduced in *Escherichia coli*. The Km and *k*cat values for the conversion of tyrosine to 4-coumaric acid were 15.6 µM and 27.7 s^{-1} respectively, and for conversion of L-phenylalanine to *trans*-cinnamic acid were 1277 µM and 15.1 s^{-1} respectively. As a consequence of the smaller Km and a slightly larger *k*cat, the enzyme shows a strong preference for tyrosine over L-phenylalanine, with a catalytic efficiency (Km/*k*cat) for tyrosine of approximately 150-fold larger than for phenylalanine. The kinetic studies established that tyrosine, and not L-phenylalanine, is the

natural substrate of the enzyme under physiological conditions. Very recently a study described the heterologous coexpression of phenylalanine ammonia lyase, cinnamate-4-hydroxylase, 4-coumarate-Coa-ligase and chalcone synthase, for the production of flavonoids in E. coli (Watts *et al.*, 2004). The simultaneous expression of all four genes, however, was not successful because of a nonfunctional cinnamate-4-hydroxylase. The substitution of phenylalanine ammonia lyase and cinnamate-4-hydroxylase by a new tyrosine ammonia lyase that was cloned from *Rhodobacter sphaeroides*, could, however, solved the problem and led to high-level production of the flavonone naringenin. Furthermore, said tyrosine ammonia lyase from *Rhodobacter sphaeroides* is also used for heterologous production of 4-coumaric acid (i.e. *para*-hydroxycinnamic acid) in *Escherichia coli* (US-A-2004059103). Evenmore, further methods for development of a biocatalyst for conversion of glucose into 4-coumaric acid are described. US-A-2004023357 discloses a tyrosine ammonia lyase from the yeast *Trichosporon cutaneum* for the production of coumaric acid in *Escherichia coli* and *Saccharomyces cerevisiae*. US-A-2001053847 describes the incorporation of the wild type PAL from the yeast *Rhodotorula glutinis* into *E. coli*, underlining the ability of the wildtype PAL to convert tyrosine directly to 4-coumaric acid. Moreover, there is also exemplification of incorporation of the wildtype PAL from the yeast *Rhodotorula glutinis*, plus a plant C4H and CPR into *E. coli* and *S. cerevisiae*. Also described is the development of a biocatalyst through mutagenesis of the wild type yeast PAL *Rhodotorula glutinis* with enhanced TAL activity (US-A-6521748). Neither of the aforementioned patents claim the incorporation of 4CL and VST for the production of resveratrol.

Recently, evidence was shown that the filamentous fungi *A. oryzae* contained the enzyme chalcone synthase (CHS) that is normally involved in the biosynthesis of flavonoids, such as naringenin, in plants (Seshime *et al.*, 2005). Indeed it was also shown that *A. oryzae* contained the major set of genes responsible for phenylpropanoid-flavonoid metabolism, i.e PAL, C4H and 4CL. However, there is no evidence that *A. oryzae* contained a stilbene synthase such as resveratrol synthase.

The present invention now provides a micro-organism having an operative metabolic pathway comprising at least one enzyme activity, said pathway producing 4-coumaric acid and producing resveratrol therefrom or an oligomeric or glycosidically-bound derivative thereof. Such a micro-organism may be naturally occurring and may be isolated by suitable screening procedures, but more preferably is genetically engineered.

Preferably, said resveratrol or derivative is produced in a reaction catalysed by an enzyme in which endogenous malonyl-CoA is a substrate, and preferably said resveratrol is produced from 4-coumaroyl-CoA.

Said resveratrol or derivative is preferably produced from 4-coumaroyl-CoA by a resveratrol synthase which is preferably expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

Generally herein, unless the context implies otherwise, references to resveratrol include reference to oligomeric or

glycosidically bound derivatives thereof, including particularly piceid.

Thus, in certain preferred embodiments, said resveratrol synthase is a resveratrol synthase (EC 2.3.1.95) from a plant belonging to the genus of *Arachis*, e.g. *A. glabatra*, *A. hypogaea*, a plant belonging to the genus of *Rheum*, e.g. *R. tataricum*, a plant belonging to the genus of *Vitus*, e.g. *V. labrusca*, *V. riparaia*, *V. vinifera*, or any one of the genera *Pinus*, *Piceea*, *Lilium*, *Eucalyptus*, *Parthenocissus*, *Cissus*, *Calochortus*, *Polygonum*, *Gnetum*, *Artocarpus*, *Nothofagus*, *Phoenix*, *Festuca*, *Carex*, *Veratrum*, *Bauhinia or Pterolobium*.

Preferably, said 4-coumaric acid is produced from *trans*-cinnamic acid, suitably by an enzyme in a reaction catalysed by said enzyme in which oxygen is a substrate, NADH or NADPH is a cofactor and NAD^+ or $NADP^+$ is a product.

Thus, said 4-coumaric acid may be produced from *trans*-cinnamic acid by a cinnamate 4-hydroxylase, which preferably is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

In certain preferred embodiments, including those referred to in the paragraphs above, said cinnamate-4-hydroxylase is a cinnamate-4-hydroxylase (EC 1.14.13.11) from a plant or a micro-organism. The plant may belong to the genus of *Arabidopsis*, e.g. *A. thaliana*, a plant belonging to the genus of *Citrus*, e.g. *C. sinensis*, *C. x paradisi*, a plant belonging to the genus of *Phaseolus*, e.g. *P. vulgaris*, a plant belonging to the genus of *Pinus*, e.g. *P. taeda*, a plant belonging to the genus of *Populus*, e.g. *P. deltoides*,

P. tremuloides, *P. trichocarpa*, a plant belonging to the genus of *Solanum*, e.g. *S. tuberosum*, a plant belonging to the genus of *Vitus*, e.g. *Vitus vinifera*, a plant belonging to the genus of *Zea*, e.g. *Z. mays*, or other plant genera e.g. *Ammi*, *Avicennia*, *Camellia*, *Camptotheca*, *Catharanthus*, *Glycine*, *Helianthus*, *Lotus*, *Mesembryanthemum*, *Physcomitrella*, *Ruta*, *Saccharum*, *Vigna*. The micro-organism might be a fungus belonging to the genus *Aspergillus*, e.g. *A. oryzae*.

Preferably, said 4-coumaric acid is produced from tyrosine in a reaction catalysed by an enzyme in which ammonia is produced and suitably, said 4-coumaric acid is produced from *tyrosine* by a L-phenylalanine ammonia lyase or a tyrosine ammonia lyase, e.g. tyrosine ammonia lyase (EC 4.3.1.5) from yeast or bacteria. Suitably, the tyrosine ammonia lyase is from the yeast *Rhodotorula rubra* or from the bacterium *Rhodobacter capsulatus*.

Optionally, said tyrosine ammonia lyase is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

Alternatively, said *trans*-cinnamic acid may be produced from L-phenylalanine in a reaction catalysed by an enzyme in which ammonia is produced and suitably said *trans*-cinnamic acid is formed from L-phenylalanine by a phenylalanine ammonia lyase.

In certain preferred embodiments, said L-phenylalanine ammonia lyase is a L-phenylalanine ammonia lyase (EC 4.3.1.5) from a plant or a micro-organism. The plant may belong to the genus of *Arabidopsis*, e.g. *A. thaliana*, a

plant belonging to the genus of *Brassica*, e.g. *B. napus*, *B. rapa*, a plant belonging to the genus of *Citrus*, e.g. *C. reticulata*, *C. clementinus*, *C. limon*, a plant belonging to the genus of *Phaseolus*, e.g. *P. coccineus*, *P. vulgaris*, a plant belonging to the genus of *Pinus*, e.g. *P. banksiana*, *P. monticola*, *P. pinaster*, *P. sylvestris*, *P. taeda*, a plant belonging to the genus of *Populus*, e.g. *P. balsamifera*, *P. deltoides*, *P. Canadensis*, *P. kitakamiensis*, *P. tremuloides*, a plant belonging to the genus of *Solanum*, e.g. *S. tuberosum*, a plant belonging to the genus of *Prunus*, e.g. *P. avium*, *P. persica*, a plant belonging to the genus of *Vitus*, e.g. *Vitus vinifera*, a plant belonging to the genus of *Zea*, e.g. *Z. mays* or other plant genera e.g. *Agastache*, *Ananas*, *Asparagus*, *Bromheadia*, *Bambusa*, *Beta*, *Betula*, *Cucumis*, *Camellia*, *Capsicum*, *Cassia*, *Catharanthus*, *Cicer*, *Citrullus*, *Coffea*, *Cucurbita*, *Cynodon*, *Daucus*, *Dendrobium*, *Dianthus*, *Digitalis*, *Dioscorea*, *Eucalyptus*, *Gallus*, *Ginkgo*, *Glycine*, *Hordeum*, *Helianthus*, *Ipomoea*, *Lactuca*, *Lithospermum*, *Lotus*, *Lycopersicon*, *Medicago*, *Malus*, *Manihot*, *Medicago*, *Mesembryanthemum*, *Nicotiana*, *Olea*, *Oryza*, *Pisum*, *Persea*, *Petroselinum*, *Phalaenopsis*, *Phyllostachys*, *Physcomitrella*, *Picea*, *Pyrus*, *Quercus*, *Raphanus*, *Rehmannia*, *Rubus*, *Sorghum*, *Sphenostylis*, *Stellaria*, *Stylosanthes*, *Triticum*, *Trifolium*, *Triticum*, *Vaccinium*, *Vigna*, *Zinnia*. The micro-organism might be a fungus belonging to the genus *Agaricus*, e.g. *A. bisporus*, a fungus belonging to the genus *Aspergillus*, e.g. *A. oryzae*, *A. nidulans*, *A. fumigatus*, a fungus belonging to the genus *Ustilago*, e.g. *U. maydis*, a bacterium belonging to the genus *Rhodobacter*, e.g. *R. capsulatus*, a yeast belonging to the genus *Rhodotorula*, e.g. *R. rubra*.

Suitably, said L-phenylalanine ammonia lyase is expressed in said micro-organism from nucleic acid coding for said enzyme

which is not native to the micro-organism.

Preferably, 4-coumaroyl-CoA is formed in a reaction catalysed by an enzyme in which ATP and CoA are substrates and ADP is a product and suitably 4-coumaroyl-CoA is formed in a reaction catalysed by a 4-coumarate-CoA ligase.

Said 4-coumarate-CoA ligase may be a 4-coumarate-CoA ligase (EC 6.2.1.12) from a plant, a micro-organism or a nematode. The plant may belong to the genus of *Abies*, e.g. *A. beshanzuensis, B. firma, B. holophylla*, a plant belonging to the genus of *Arabidopsis*, e.g. *A. thaliana*, a plant belonging to the genus of *Brassica*, e.g. *B. napus, B. rapa, B.oleracea*, a plant belonging to the genus of *Citrus*, e.g. *C. sinensis*, a plant belonging to the genus of *Larix*, e.g. *L. decidua, L. gmelinii, L. griffithiana, L. himalaica, L. kaempferi, L. laricina, L. mastersiana, L. occidentalis, L. potaninii, L. sibirica, L. speciosa*, a plant belonging to the genus of *Phaseolus*, e.g. *P. acutifolius, P. coccineus*, a plant belonging to the genus of *Pinus*, e.g. *P. armandii P. banksiana, P. pinaster*, a plant belonging to the genus of *Populus*, e.g. *P. balsamifera, P. tomentosa, P. tremuloides*, a plant belonging to the genus of *Solanum*, e.g. *S. tuberosum*, a plant belonging to the genus of *Vitus*, e.g. *Vitus vinifera*, a plant belonging to the genus of *Zea*, e.g. *Z. mays*, or other plant genera e.g. *Agastache, Amorpha, Cathaya, Cedrus, Crocus, Festuca, Glycine, Juglans, Keteleeria, Lithospermum, Lolium, Lotus, Lycopersicon, Malus, Medicago, Mesembryanthemum, Nicotiana, Nothotsuga, Oryza, Pelargonium, Petroselinum, Physcomitrella, Picea, Prunus, Pseudolarix, Pseudotsuga, Rosa, Rubus, Ryza, Saccharum, Suaeda, Thellungiella, Triticum, Tsuga.* The micro-organism might be a filamentous fungi belonging to the

genus *Aspergillus*, e.g. *A. flavus*, *A. nidulans*, *A. oryzae*, *A. fumigatus*, a filamentous fungus belonging to the genus *Neurospora*, e.g. *N. crassa*, a fungus belonging to the genus Yarrowia, e.g. *Y. lipolytica*, a fungus belonging to the genus of *Mycosphaerella*, e.g. *M. graminicola*, a bacterium belonging to the genus of *Mycobacterium*, e.g. *M. bovis*, *M. leprae*, *M. tuberculosis*, a bacterium belonging to the genus of *Neisseria*, e.g. *N. meningitidis*, a bacterium belonging to the genus of *Streptomyces*, e.g. *S. coelicolor*, a bacterium belonging to the genus of *Rhodobacter*, e.g. *R. capsulatus*, a nematode belonging to the genus *Ancylostoma*, e.g. *A. ceylanicum*, a nematode belonging to the genus *Caenorhabditis*, e.g. *C. elegans*, a nematode belonging to the genus *Haemonchus*, e.g. *H. contortus*, a nematode belonging to the genus *Lumbricus*, e.g. *L. rubellus*, a nematode belonging to the genus *Meilodogyne*, e.g. *M. hapla*, a nematode belonging to the genus *Strongyloidus*, e.g. *S. rattii*, *S. stercoralis*, a nematode belonging to the genus *Pristionchus*, e.g. *P. pacificus*.

Optionally, a NADPH:cytochrome P450 reductase (CPR) has been recombinantly introduced into said micro-organism. This may be a plant CPR introduced into a non-plant micro-organism. Alternatively, a native NADPH:cytochrome P450 reductase (CPR) has been overexpressed in said micro-organism.

In certain preferred embodiments, including those referred to in the paragraphs above, said NADPH:cytochrome P450 reductase is a NADPH:cytochrome P450 reductase (EC 1.6.2.4) from a plant belonging to the genus of *Arabidopsis*, e.g. *A. thaliana*, a plant belonging to the genus of *Citrus*, e.g. *C. sinensis*, *C. x paradisi*, a plant belonging to the genus of *Phaseolus*, e.g. *P. vulgaris*, a plant belonging to the genus

of *Pinus*, e.g. *P. taeda*, a plant belonging to the genus of *Populus*, e.g. *P. deltoides*, *P. tremuloides*, *P. trichocarpa*, a plant belonging to the genus of *Solanum*, e.g. *S. tuberosum*, a plant belonging to the genus of *Vitus*, e.g. *Vitus vinifera*, a plant belonging to the genus of *Zea*, e.g. *Z. mays*, or other plant genera e.g. *Ammi*, *Avicennia*, *Camellia*, *Camptotheca*, *Catharanthus*, *Glycine*, *Helianthus*, *Lotus*, *Mesembryanthemum*, *Physcomitrella*, *Ruta*, *Saccharum*, *Vigna*.

Whilst the micro-organism may be naturally occurring, preferably at least one copy of at least one genetic sequence encoding a respective enzyme in said metabolic pathway has been recombinantly introduced into said micro-organism.

Additionally or alternatively to introducing coding sequences coding for a said enzyme, one may provide one or more expression signals, such as promoter sequences, not natively associated with said coding sequence in said organism. Thus, optionally, at least one copy of a genetic sequence encoding a tyrosine ammonia lyase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism, and/or at least one copy of a genetic sequence encoding a L-phenylalanine ammonia lyase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

Optionally, at least one copy of a genetic sequence encoding cinnamate 4-hydroxylase, whether native or not, is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

Optionally, at least one copy of a genetic sequence encoding a 4-coumarate-CoA ligase, whether native or not, is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

Optionally, at least one copy of a genetic sequence encoding a resveratrol synthase, whether native or not, is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

Expression signals include nucleotide sequences located upstream (5′ non-coding sequences), within, or downstream (3′ non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Such sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

In certain aspects the invention provides a metabolically engineered micro-organism having an operative metabolic pathway in which a first metabolite is transformed into a second metabolite in a reaction catalysed by a first enzyme, said reaction step producing ammonia, and in which said second metabolite is transformed into a third metabolite in a reaction catalysed by a second enzyme, in which oxygen is a substrate, NADPH or NADH is a cofactor and $NADP^+$ or NAD^+ is a product, and in which said third metabolite is transformed into a fourth metabolite in a reaction catalysed by a third enzyme in which ATP and CoA is a substrate, and ADP is a product, and in which said fourth metabolite is transformed

into a fifth metabolite in a reaction catalysed by a fourth enzyme in which endogenous malonyl-CoA is a substrate.

The present invention also provides a metabolically engineered micro-organism having an operative metabolic pathway in which a first metabolite is transformed into a said third metabolite catalysed by a first enzyme, said reaction step producing ammonia, without the involvement of said second enzyme, and in which said third metabolite is transformed into a said fourth metabolite in a reaction catalysed by a said third enzyme in which ATP and CoA is a substrate, and ADP is a product, and in which said fourth metabolite is transformed into a said fifth metabolite in a reaction catalysed by a said fourth enzyme in which endogenous malonyl-CoA is a substrate.

The micro-organisms described above include ones containing one or more copies of an heterologous DNA sequence encoding phenylalanine ammonia lyase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding cinnamate-4-hydroxylase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding 4-coumarate-CoA-ligase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding resveratrol synthase operatively associated with an expression signal.

They include also ones lacking cinnamate-4-hydroxylase activity, and containing one or more copies of a heterologous DNA sequence encoding tyrosine ammonia lyase operatively associated with an expression signal, and

containing one or more copies of an heterologous DNA sequence encoding 4-coumarate-CoA-ligase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding resveratrol synthase operatively associated with an expression signal.

In the present context the term "micro-organism" relates to microscopic organisms, including bacteria, microscopic fungi, including yeast.

More specifically, the micro-organism may be a fungus, and more specifically a filamentous fungus belonging to the genus of *Aspergillus*, e.g. *A. niger*, *A. awamori*, *A. oryzae*, *A. nidulans*, a yeast belonging to the genus of *Saccharomyces*, e.g. *S. cerevisiae*, *S. kluyveri*, *S. bayanus*, *S. exiguus*, *S. sevazzi*, *S. uvarum*, a yeast belonging to the genus *Kluyveromyces*, *e.g. K. lactis K. marxianus var. marxianus*, *K. thermotolerans*, a yeast belonging to the genus *Candida*, *e.g. C. utilis C. tropicalis*, *C.albicans*, *C. lipolytica*, *C. versatilis*, a yeast belonging to the genus *Pichia*, *e.g. P. stipidis*, *P. pastoris*, *P. sorbitophila*, or other yeast genera, *e.g. Cryptococcus*, *Debaromyces*, *Hansenula*, *Pichia*, *Yarrowia*, *Zygosaccharomyces* or *Schizosaccharomyces*. Concerning other micro-organisms a non-exhaustive list of suitable filamentous fungi is supplied: a species belonging to the genus *Penicillium*, *Rhizopus*, *Fusarium*, *Fusidium*, *Gibberella*, *Mucor*, *Mortierella*, *Trichoderma*.

Concerning bacteria a non-exhaustive list of suitable bacteria is given as follows: a species belonging to the genus *Bacillus*, a species belonging to the genus

Escherichia, a species belonging to the genus *Lactobacillus*, a species belonging to the genus *Lactococcus*, a species belonging to the genus *Corynebacterium*, a species belonging to the genus *Acetobacter*, a species belonging to the genus *Acinetobacter*, a species belonging to the genus *Pseudomonas*, etc.

The preferred micro-organisms of the invention may be *S. cerevisiae*, *A. niger*, *A. oryzae*, *E. coli*, *L. lactis* or *B. subtilis*.

The constructed and engineered micro-organism can be cultivated using commonly known processes, including chemostat, batch, fed-batch cultivations, etc.

Thus, the invention includes a method for producing resveratrol or an oligomeric or glycosidically-bound derivative thereof comprising contacting a non-plant cell with a carbon substrate in the substantial absence of an external source of 4-coumaric acid, said cell having the capacity to produce resveratrol or an oligomeric or glycosidically-bound derivative thereof under the conditions, in which the micro-organism may be selected from the group consisting of fungi and bacteria, especially yeast.

Said carbon substrate is optionally selected from the group of fermentable carbon substrates consisting of monosaccharides, oligosaccharides and polysaccharides, e.g. glucose, fructose, galactose, xylose, arabinose, mannose, sucrose, lactose, erythrose, threose, and/or ribose. Said carbon substrate may additionally or alternatively be selected from the group of non-fermentable carbon substrates

including ethanol, acetate, glycerol, and/or lactate. Said non-fermentable carbon substrate may additionally or alternatively be selected from the group of amino acids and may be phenylalanine and/or tyrosine.

In an alternative aspect, the invention includes a method for producing resveratrol or an oligomeric or glycosidically-bound derivative thereof through heterologous expression of nucleotide sequences encoding phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate-CoA ligase and resveratrol synthase and also a method for producing resveratrol through heterologous expression of nucleotide sequences encoding tyrosine ammonia lyase, 4-coumarate-CoA ligase and resveratrol synthase.

Resveratrol or an oligomeric or glycosidically-bound derivative thereof so produced may be used as a nutraceutical in a dairy product or a beverage such as beer.

Resveratrol produced according to the invention may be *cis*-resveratrol or *trans*-resveratrol, but it is to be expected that the *trans*- form will normally predominate.

BRIEF DESCRIPTION OF THE DRAWINGS

To assist in the ready understanding of the above decription of the invention reference has been made to the accompanying drawings in which:

Figure 1 shows the chemical structure of *trans*-resveratrol;

Figure 2 shows the phenylpropanoid pathway utilising phenylalanine ammonia lyase acting on L-phenylalanine; and

Figure 3 shows the alternative pathway utilising phenylalanine ammonia lyase acting on L-tyrosine.

Figure 4 shows the HPLC-chromatograms of extracts of *S. cerevisiae* strains FSSC-PALC4H4CLVST, FSSC-TAL4CLVST, grown on 100 g/l galactose. A chromatogram of 60 nanogram of pure resveratrol is included.

Figure 5 shows the UV absorption spectrum for pure *trans*-resveratrol and *trans*-resveratrol produced by *S. cerevisiae* strain FSSC-PALC4H4CLVST, grown on 100 g/l galactose.

Figure 6 shows the HPLC-chromatograms of extracts from *E. coli* strains FSEC-TAL4CLVST and FSEC-control, grown on 50 g/l glucose.

Figure 7 shows the HPLC-chromatograms of extracts from *E. coli* strains FSEC-TAL4CLVST and FSEC-control, grown on 50 g/l glucose with addition of 20 mg/l coumaric acid. The UV absorption spectrum for *trans*-resveratrol produced in strain FSEC-TAL4CLVST is included.

The invention will be further described and illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Isolation of genes encoding PAL, TAL, C4H, CPR, 4CL, and VST

Phenylalanine ammonia lyase (PAL2) (Cochrane *et al.*, 2004; SEQ ID NO: 1, 2), cinnamate 4-hydroxylase (C4H) (Mizutani *et al.*, 1997; SEQ ID NO: 3, 4) and 4-coumarate:CoenzymeA ligase (4CL1) (Hamberger and Hahlbrock 2004; Ehlting *et al.*, 1999; SEQ ID NO: 5, 6) were isolated via PCR from *A. thaliana* cDNA (BioCat, Heidelberg, Germany) using the primers in table 1. PAL2 and 4CL1 were chosen amongst several *A. thaliana* homologues due to favourable kinetic parameters towards cinnamic acid and coumaroyl-CoA, respectively (Cochrane *et al.*,, 2004; Hamberger and Hahlbrock 2004; Ehlting *et al.*, 1999).

The coding sequence of resveratrol synthase (VST) from *Rheum tataricum* (Samappito *et al.*, 2003; SEQ ID NO: 7, 8) and tyrosine ammonia lyase (TAL) from *Rhodobacter capsulatus* (Kyndt *et al.*, 2002; SEQ ID NO: 11, 12) were codon optimized for expression in *S. cerevisiae* using the online service backtranslation tool at www.entelechon.com, yielding sequence SEQ ID NO: 9, 10 and SEQ ID NO: 13, 14 respectively. Oligos for the synthetic gene assembly were constructed at MWG Biotech and the synthetic gene was assembled by PCR using a slightly modified method protocol of from Martin *et al.* (2003) described below.

Table 1. Primers and restriction sites for the amplification of genes

Primer for amplification of gene* (Restriction sites are underlined)	Gene	Restriction site: primer	Restriction site: vector
5′-CGGAATTCTCATGGATCAAATCGAAGCAATGTT	PAL2	EcoR1	EcoR1
5′-CGACTAGTTTAGCAAATCGGAATCGGAGC	PAL2	Spe1	Spe1
5′-CGCTCGAGAT ATGGACCTCCTCTTGCTGGA	C4H	Xho1	Xho1
5′-CGGGTACCTTAACAGTTCCTTGGTTTCATAAC	C4H	Kpn1	Kpn1
5′-GCTCTAGACCT ATGGCGCCACAAGAACAAGCAGTTT	4CL1	Xba1	Spe1
5′-GCGGATCCCCT TCACAATCCATTTGCTAGTTT TGCC	4CL1	BamH1	BglII
5′-CC GGATCCAAATGGCCCCAGAAGAGAGCAGG	VST	BamH1	BamH1
5′-CG CTCGAGTTAAGTGATCAATGGAACCGAAGACAG	VST	Xho1	Xho1
5′-CCGAATTCCCATGACCCTGCAATCTCAAACAGCTAAAG	TAL	EcoR1	EcoR1
5′-CCACTAGTTTAAGCAGGTGGATCGGCAGCT	TAL	Spe1	Spe1
5′-CCCTCGAGATCATGCCGTTTGGAATAGACAACACCGA	CPR1	Xho1	Xho1
5′-CCAAGCTTATCGGGCTGATTACCAGACATCTTCTTG	CPR1	HindIII	HindIII
5′-CCGGATCCCCATGTCCTCTTCTTCTTCTTCGTCAAC	AR2	Bamh1	Bamh1
5′-CCCTCGAGGTGAGTGTGTGGCTTCAATAGTTT CG	AR2	Xho1	Xho1

* SEQ ID Nos 19-32

Primers from MWG for the assembly of the synthetic gene were dissolved in milliQ-water to a concentration of 100 pmole/µl. An aliquot of 5 µl of each primer was combined in a totalmix and then diluted 10-fold with milliQ water. The gene was assembled via PCR using 5 µl diluted totalmix per 50 µl as template for fusion DNA polymerase (Finnzymes). The PCR programme was as follows: Initial 98 °C for 30 s., and then 30 cycles with 98 °C for 10 s., 40 °C for 1 min. and 72 °C at 1 min./1000 basepairs, and a final 72 °C for 5 min. From the resulting PCR reaction, 20 µl was purified on 1% agarose gel. The result was a PCR smear and the regions around the wanted size were cut out from agarose gel and purified using the QiaQuick Gel Extraction Kit (Qiagen). A final PCR with the outer primers (for TAL and VST) in table 1 rendered the required TAL and VST genes. Point mutations were corrected using either the Quickchange site directed

mutagenesis II kit (Stratagene, La Jolla, CA), or using PCR from overlapping error free DNA stretches from several different *E. coli* subclones.

NADPH:Cytochrome P450 reductase (CPR) from *A. thaliana* (AR2) (Mizutani and Ohta, 1998; SEQ ID NO: 17, 18) and from *S. cerevisiae* (CPR1) (Aoyama *et al.*, 1978; SEQ ID NO: 15, 16), were isolated from *A. thaliana* cDNA (BioCat, Heidelberg, Germany) and *S. cerevisae* genomic DNA, respectively, using the primers in table 1.

Example 2

Construction of a yeast vector for expression of PAL

The gene encoding PAL, isolated as described in example 1, was reamplified by PCR using forward- and reverse primers, with 5′ overhangs containing EcoR1 and Spe1 restriction sites (table 1). The amplified PAL PCR product was digested with EcoR1/Spe1 and ligated into EcoR1/Spe1 digested pESC-URA vector (Stratagene), resulting in vector pESC-URA-PAL. The sequence of the gene was verified by sequencing of two different clones.

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Example 3

Construction of a yeast vector for expression of PAL and C4H

The gene encoding C4H, isolated as described in example 1, was amplified by PCR using the forward- and reverse primers, with 5′ overhangs containing Xho1 and Kpn1 restriction sites. The amplified C4H PCR-product was digested with Xho1/Kpn1 and ligated into similarly digested pESC-URA-PAL vector. The resulting plasmid, pESC-URA-PAL-C4H, contained

the genes encoding PAL and C4H under the control of the divergent GAL1/GAL10 promoter. The sequence of the gene encoding C4H was verified by sequencing of two different clones.

Example 4

Construction of a yeast vector for expression of 4CL

The gene encoding 4CL was isolated as described in example 1. The amplified 4CL PCR-product was digested with Xba1/BamH1 and ligated into Spe1/BglII digested pESC-TRP vector (Stratagene), resulting in vector pESC-TRP-4CL.
Two different clones of pESC-TRP-4CL were sequenced to verify the sequence of the cloned gene.

Example 5

Construction of a yeast vector for expression of 4CL and VST

The gene encoding VST was isolated as described in example 1. The amplified synthetic VST gene was digested with BamH1/Xho1 and ligated into BamH1/Xho1 digested pESC-TRP-4CL (example 4). The resulting plasmid, pESC-TRP-4CL-VST, contained the genes encoding 4CL and VST under the control of the divergent GAL1/GAL10 promoter. The sequence of the gene encoding VST was verified by sequencing of two different clones of pESC-TRP-4CL-VST.

Example 6

Construction of a yeast vector for expression of TAL

The gene encoding TAL was isolated as described in example 1. The amplified synthetic TAL gene was digested with

EcoR1/Spe1 and ligated into EcoR1/Spe1-digested pESC-URA vector. The resulting plasmid, pESC-URA-TAL, contained the gene encoding for TAL under the control of the divergent GAL1/GAL10 promoter. The sequence was verified by sequencing of two different clones of pESC-URA-TAL.

Example 7

Construction of a yeast vector for overexpression of S. cerevisiae endogenous CPR

The gene encoding CPR from *S. cerevisiae* (CPR1) was isolated as described in example 1. The amplified CPR1 gene was digested with Xho1/HindIII and ligated into Xho1/HindIII-digested pESC-LEU vector (Stratagene), resulting in vector pESC-LEU-CPR1. The sequence was verified by sequencing of two different clones of pESC-LEU-CPR1.

Example 8

Construction of a yeast vector for overexpression of A. thaliana CPR (AR2)

The gene encoding CPR from *A. thaliana* (AR2) was isolated as described in example 1. The amplified AR2 gene was digested with BamH1/Xho1 and ligated into BamH1/Xho1 digested pESC-LEU vector (Stratagene), resulting in vector pESC-LEU-AR2. The sequence was verified by sequencing of two different clones of pESC-LEU-AR2.

Example 9

Expression of the pathway to resveratrol in the yeast S. cerevisiae using PAL, C4H, 4CL and VST

Yeast strains containing the appropriate genetic markers were transformed with the vectors described in examples 2, 3, 4, 5, 6, 7 and 8, separately or in combination. The transformation of the yeast cell was conducted in accordance with methods known in the art, for instance, by using competent cells or by electroporation (see, e.g., Sambrook *et al.*, 1989). Transformants were selected on medium lacking uracil and/or tryptophan and streak purified on the same medium.

S. cerevisiae strain CEN.PK 113-5D (MATa ura3) was transformed separately with the vector pESC-URA-PAL (example 2), yielding the strain FSSC-PAL, and with pESC-URA-PAL-C4H (example 3), resulting in the strain FSSC-PALC4H. *S. cerevisiae* strain FS01267 (MATa trp1 ura3) was co-transformed with pESC-URA-PAL-C4H and pESC-TRP-4CL (example 4), and the transformed strain was named FSSC-PALC4H4CL. The same strain was also co-transformed with pESC-URA-PAL-C4H and pESC-TRP-4CL-VST (example 5), resulting in the strain FSSC-PALC4H4CLVST.

Example 10

Expression of the pathway to resveratrol in S. cerevisiae using TAL, 4CL and VST.

S. cerevisiae strain CEN.PK 113-5D (MATa ura3) was transformed separately with the vector pESC-URA-TAL (example

6), yielding the strain FSSC-TAL. *S. cerevisiae* strain FS01267 (MATa trp1 ura3) was co-transformed with pESC-URA-TAL (example 6) and pESC-TRP-4CL (example 4), and the transformed strain was named FSSC-TAL4CL. The same strain was also co-transformed with pESC-URA-TAL and pESC-TRP-4CL-VST (example 5), resulting in the strain FSSC-TAL4CLVST. Transformants were selected on medium lacking uracil and or tryptophan and streak purified on the same medium.

Example 11

Expression of the pathway to resveratrol in S.cerevisiae with overexpressed endogenous CPR.

S. cerevisiae strain FS01277 (MATa ura3 leu2 trp1) was co-transformed with vectors pESC-URA-PAL-C4H (example 3), pESC-TRP-4CL (example 4), and pESC-LEU-CPR1 (example 7). The transformed strain was named FSSC-PALC4H4CLVSTCPR. Transformants were selected on medium lacking uracil and/or tryptophan and streak purified on the same medium.

Example 12

Expression of the pathway to resveratrol in S.cerevisiae with overexpressed A. thaliana CPR (AR2).

S. cerevisiae strain FS01277 (MATa ura3 leu2 trp1) was co-transformed with vectors pESC-URA-PAL-C4H (example 3), pESC-TRP-4CL (example 4), and pESC-LEU-AR2 (example 8). The transformed strain was named FSSC-PALC4H4CLVSTAR2. Transformants were selected on medium lacking uracil and or tryptophan and streak purified on the same medium.

Example 13

Fermentation with recombinant yeast strains in shake flasks

The recombinant yeast strains were inoculated from agar plates with a sterile inoculation loop and grown in 200 ml defined mineral medium (Verduyn et al, 1992) that contained vitamins, trace elements, 5 g/l glucose and 40 g/l or 100 g/l galactose. The 500 ml stoppered shake flasks were incubated for three days at 30 °C and 160 rpm.

Example 14

Extraction of resveratrol

Cells were harvested by centrifugation 5000 g for 5 minutes. An aliquot of 50 ml of supernatant was extracted once with 20 ml ethyl acetate. The ethyl acetate was freeze dried and the dry product redissolved in 0.7 ml methanol and filtered into HPLC vials.

The cell pellet from 200 ml medium was dissolved in 1 to 2 ml water and divided into 3 fastprep tubes and broken with glass beads. The crude extracts from the three tubes were pooled into 10 ml 100 % methanol in a 50 ml sartorius tube and extracted on a rotary chamber for 48 hours in a dark cold room at 4 °C. After 48 hours the cell debris was removed via centrifugation for 5 min. at 5000 g and the methanol was removed by freeze-drying overnight. The dried residue was redissolved in 1 ml phosphate-citrate buffer pH 5.4 and 10 units *beta*-glucosidase from almonds was added (Sigma) to release resveratrol from putatively glucoside-bound forms. The mixture was incubated for three hours at 37 °C and then extracted twice with 1 ml ethyl acetate. The combined ethyl

acetate was freeze dried and the dry residue was redissolved in 0.7 ml methanol and filtered into HPLC vials.

Example 15

Analysis of resveratrol

Thin layer chromatography

A method based upon thin layer chromatography that enabled the quick separation of cinnamic, coumaric and resveratrol on the same TLC-plate was developed for quick screening analysis. An aliquot of 1 ml culture containing both cells and supernatant were extracted with 500 microliter ethyl acetate and centrifuged for 30 s. at 13000 rpm with a microcentrifuge. The ethyl acetate was dried and redissolved in methanol. The extracts were analyzed on Silica G plates (0.2 mm Alugram SIL G/UV_{254}, Macherey-Nagel) containing a fluorescent indicator. The mobile phase was a mixture of chloroform, ethyl acetate and formic acid (25:10:1).

HPLC

For quantitative analysis of cinnamic acid, coumaric acid, and resveratrol, samples were subjected to separation by high-performance liquid chromatography (HPLC) Agilent Series 1100 system (Hewlett Packard) prior to uv-diode-array detection at λ = 306 nm. A Phenomenex (Torrance, CA, USA) Luna 3 micrometer C18 (100 X 2.00 mm) column was used at 40 °C. As mobile phase a gradient of acetonitrile and milliq water (both containing 50 ppm trifluoroacetic acid) was used at a flow of 0.4 ml/min. The gradient profile was linear from 15 % acetonitrile to 100 % acetonitrile over 20 min.

The elution times were approximately 3.4 min. for coumaric acid, 5.5 min. for free *trans*-resveratrol and 6.8 min. for cinnamic acid.

Pure resveratrol standard was purchased from Cayman chemical company, whereas pure coumaric acid and cinnamic acid standards were purchased from and Sigma.

Results

Strains FSSC-PALC4H4CLVST and FSSC-TAL4CLVST, were cultivated on 100 g/l galactose as described in example 13, and analyzed for their content of intracellular resveratrol according to example 14 and 15. Additionally, a control strain FSSC-control was included that contained the empty vectors pESC-URA and pESC-TRP only. The HPLC-analysis showed that strains FSSC-PALC4H4CLVST and FSSC-TAL4CLVST contained a component with a retention time of 5.5 min. that was identical to *trans*-resveratrol (figure 4). Said result was confirmed by the UV absorption spectra that were similar to the absorption spectrum of pure *trans*-resveratrol (figure 5) as well, with a λ_{max} of approximately 306 nm.

The results, therefore, demonstrated the presence of an active phenyl-propanoid pathway in *S. cerevisiae* that led to *in vivo* production of *trans*-resveratrol. The production of resveratrol can most likely be improved by cultivating the strains under well-defined growth conditions in batch- and continuous cultures, and/or optimizing the expression/activities of the individual enzymes.

Example 16

Construction of a bacterial vector for expression of TAL in Escherichia coli.

The gene encoding TAL, isolated as described in Example 1, was reamplified by PCR from the plasmid pESC-URA-TAL (example 6) using the forward primer 5′-CCG CTCGAG CGG ATG ACC CTG CAA TCT CAA ACA GCT AAA G-3′ SEQ ID NO 33 and the reverse primer 5′-GC GGATCC TTA AGC AGG TGG ATC GGC AGC T-3′ SEQ ID NO 34 with 5′ overhangs containing the restriction sites XhoI and BamHI, respectively. The introduction of restriction sites at the 5′ and 3′ ends of the gene allowed ligation of the restricted PCR product into a pET16b vector (Novagen), digested with XhoI and BamHI to yield pET16b-TAL. The pET16b vector contained both the ampicillin resistance gene, and the T7 promoter. Hence, above procedure resulted in a vector with an antibiotic selection marker that contained the gene encoding for TAL under the control of the T7 promoter. The sequence of the gene encoding TAL was verified by sequencing of one clone of pET16b-TAL.

Example 17

Construction of a bacterial vector for expression of 4CL and VST in Escherichia coli.

The gene encoding VST, isolated as described in example 1, was cut out with the restriction enzymes BamHI and XhoI from the digested plasmid pESC-TRP-4CL-VST (example 5), which contains the genes encoding 4CL and VST. The VST gene was ligated into a pET26b vector (Novagen), containing the kanamycin resistance gene, digested with BamHI and SalI to yield pET26b-VST. The restriction enzymes XhoI and SalI have

compatible ends, which enabled proper ligation. The pET26b vector contained both the kanamycin resistance gene, and the T7 promoter. Hence, above procedure resulted in a vector with an antibiotic selection marker that contained the gene encoding for VST under the control of the T7 promoter.

The gene encoding for 4CL, isolated as described in example 1, was reamplified by PCR from the plasmid pESC-URA-4CL-VST (example 5) using the forward primer 5′-TG CCATGG CA ATGGCGCCAC AAGAACAAGC AGTTT-3′ SEQ ID NO 35 and the reverse primer 5′-GC GGATCC CCT TCA CAA TCC ATT TGC TAG TTT TGCC-3′ SEQ ID NO 36 with 5′ overhangs containing the restriction sites NcoI and BamHI, respectively. The introduction of restriction sites at the 5′ and 3′ ends of the gene allowed ligation of the restricted PCR product into a pET16b vector (Novagen) digested with NcoI and BamHI. The resulting plasmid, pET16b-4CL, contained the gene encoding for 4CL under the control of the T7 promoter. Both the T7 promoter and the gene encoding for 4CL were reamplified as one fragment by PCR from the plasmid pET16b-4CL using the forward primer 5′-TT GCGGCCGC AAA TCT CGA TCC CGC GAA ATT AAT ACG-3′ SEQ ID NO 37 and the reverse primer 5′-CG CTCGAG CCT TCA CAA TCC ATT TGC TAG TTT TGCC-3′ SEQ ID NO 38 with 5′ overhangs, containing the restriction sites NotI and XhoI, respectively. The introduction of restriction sites at the 5′ and 3′ ends of the DNA fragment allowed ligation of the restricted PCR product into the plasmid pET26b-VST that was digested with NotI and XhoI before ligation. The resulting plasmid, pET26b-VST-4CL, contained the two genes 4CL and VST that each were under control of an individual T7 promoter.

Example 18

Expression of the pathway to resveratrol in Escherichia coli, using TAL, 4CL and VST.

The transformation of the bacterial cell was conducted in accordance with methods known in the art, for instance, by using competent cells or by electroporation (see, e.g., Sambrook *et al.*, 1989). The *E. coli* strain BL21 (DE3) (Novagen) was co-transformed with the two vectors pET16b-TAL (example 16) and pET26b-VST-4CL (Example 17), resulting in strain FSEC-TAL4CLVST. In addition, *E. coli* strain BL21 (DE3) was co-transformed with the two empty vectors pET16b (Novagen) and pET26b (Novagen), resulting in strain FSEC-control, which was used as a control strain. Transformants were selected on Luria-Bertani (LB) medium with 100 µg/ml ampicillin and 60 µg/ml kanamycin.

Example 19

Fermentation with recombinant Escherichia coli strains in shake flasks.

Pre-cultures of *Escherichia coli* BL21 (DE3) were grown in glass tubes at 160 rpm and 37 °C in 7 ml of LB medium containing 100 µg/ml ampicillin and 60 µg/ml kanamycin. Exponentially growing precultures were used for inoculation of 500 ml baffled shake flasks that contained 200 ml LB medium supplemented with 50 g/l glucose, 5 g/l K_2HPO_4, 80 µg/ml ampicilin and 50 µg/ml kanamycin, which were incubated at 160 rpm and 37 °C. After 5 hours, isopropyl β-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, as an inducer of the T7 promoter that was in front of each of the three genes TAL, 4CL and VST. After an incubation period of 48 hours at 37 °C, the cells

were harvested and subjected to extraction procedures and analysed for the presence of produced resveratrol.

Example 20

Extraction and analysis of resveratrol in Escherichia coli.

Extraction and analysis was performed using the methods as described in example 14 and 15.

Results

Strain FSEC-TAL4CLVST and FSEC-control, were cultivated on 50 g/l glucose as described in example 19, and analyzed for their content of intracellular resveratrol according to example 14 and 15. The HPLC-analysis showed that strain FSEC-TAL4CLVST did contain considerable amounts of a component with a retention time of 3.4 min., which is identical to coumaric acid (figure 6). However, the extract did not contain a component that eluted at the same time as *trans*-resveratrol. Said result, therefore, indicated that the tyrosine ammonia lyase (TAL) was active indeed, but did not lead to production of detactable amounts of resveratrol. The lack of resveratrol formation, however, could be the result of; i) a non-functional coumarate-CoA ligase (4CL); ii) a non-functional resveratrol synthase (VST); iii) too low levels of coumaric acid, caused by either non-optimal cultivation conditions, or non-optimal expression/actitvity of TAL, or branching of coumaric acid into other products. To evaluate said hypotheses, the strains were grown on similar media as described in example 19 but now in the presence of 20 mg/l of coumaric acid. The subsequent HPLC-analysis of extracts of FSEC-TAL4CLVST indeed showed a cluster of peaks around the same retention time as *trans*-

resveratrol, which was not observed in extracts of FS-control (figure 6). Indeed, the UV absorption spectrum of the peak with a retention time of 5.5 min. was similar to the spectrum of pure *trans*-resveratrol (figure 7), whereas no such spectrum could be obtained for peaks in the control strain. The results, therefore, strongly suggest the presence of an active phenylpropanoid pathway in *Escherichia coli*, which can lead to production of resveratrol. Most likely the production of resveratrol without addition of coumaric acid can be achieved by cultivating the strains under well-defined growth conditions in batch- and continuous cultures, and/or optimizing the expression/activities of the individual enzymes.

Example 21

Construction of a bacterial vector for expression of PAL and C4H in Lactococcus lactis.

The plasmid pSH71 and derivatives thereof, which is used in the following examples, is a bifunctional shuttle vector with multiple origins of replication from *Escherichia coli* and *Lactococcus lactis*. With that, the host range specificity traverses *Escherichia coli* and other species of lactic acid bacteria. Though transformations in *Lactoccus lactis* usually proceed without problems, putative difficult transformations in other species of lactic acid bacteria can, therefore, be overcome by using *Escherichia coli* as an intermediate host for the construction of recombinant plasmids. The plasmid contains one or more marker genes to allow the microorganism that harbour them to be selected from those which do not. The selection system that is used for *Lactococcus lactis* is based upon dominant markers, e.g. resistance against erythromycin and chloramphenicol, but

systems based upon genes involved in carbohydrate metabolism, peptidases and food grade markers, have also been described. In addition, the plasmid contains promoter- and terminator sequences that allow the expression of the recombinant genes. Suitable promoters are taken from genes of *Lactococcus lactis e.g. lacA*. Furthermore, the plasmid contains suitable unique restriction sites to facilitate the cloning of DNA fragments and subsequent identification of recombinants.

In the examples below the plasmid contains either the erythromycine resistance gene, designated as pSH71-ERY^r, or the chloramphenicol resistance gene, designated as pSH71-CM^r

The gene encoding PAL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-PAL-C4H (example 3), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pSH71-ERY^r vector that contains the *lacA* promoter from *Lactococcus lactis*. The resulting plasmid, pSH71-ERY^r-PAL, contains the gene encoding PAL under the control of the *lacA* promoter from *Lactococcuss lactis*.

The gene encoding C4H, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-PAL-C4H (example 3) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pSH71-CM^r vector to yield pSH71-CM^r-C4H. The *lacA* promoter and the gene encoding C4H are reamplified as one fragment by PCR from the plasmid pSH71-CM^r-C4H using forward- and reverse primers, with 5′ overhangs containing

suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pSH71-ERY^r-PAL. The resulting plasmid, pSH71-ERY^r-PAL-C4H, contains the genes encoding PAL and C4H that are each under the control of an individual *lacA* promoter. The sequence of the genes encoding PAL and C4H is verified by sequencing of two different clones of pSH71-ERY^r-PAL-C4H.

Example 22

Construction of a bacterial vector for expression of TAL in Lactococcus lactis.

The gene encoding for TAL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-TAL (example 6) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pSH71-ERY^r vector. The resulting plasmid, pSH71-ERY^r-TAL, contains the gene encoding for TAL under the control of the *lacA* promoter from *Lactococcus lactis*. The sequence of the gene encoding for TAL is verified by sequencing of two different clones of pSH71-ERY^r-TAL.

Example 23

Construction of a bacterial vector for expression of 4CL and VST in Lactococcus lactis.

The gene encoding 4CL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-TRP-4CL-VST

(example 5), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pSH71-CM^r vector. The resulting plasmid, pSH71-CM^r-4CL, contains the gene encoding for 4CL under the control of the *lacA* promoter from *Lactobacillus lactis*.

The gene encoding VST, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-TRP-4CL-VST (example 5) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pSH71-ERY^r vector. The resulting plasmid, pSH71-ERY^r-VST, contains the gene encoding VST under the control of the *lacA* promoter from *Lactococcus lactis*. The *lacA* promoter and the gene encoding VST are reamplified as one fragment by PCR from the plasmid pSH71-ERY^r-VST using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pSH71-CM^r-4CL. The resulting plasmid, pSH71-CM^r-4CL-VST, contains the genes encoding 4CL and VST that are each under the control of their individual *lacA* promoter. The sequence of the genes encoding 4CL and VST is verified by sequencing of two different clones of pSH71-CM^r-4CL-VST.

Example 24

Expression of the pathway to resveratrol in Lactococcus lactis

Lactococcus lactis strains are transformed with the vectors described in examples 21, 22 and 23, separately or in combination. The transformation of the bacterial cell is conducted in accordance with methods known in the art, for instance, by using competent cells or by electroporation (see, e.g., Sambrook *et al.*, 1989). Transformants are selected on medium containing the antibiotics erythromycin and chloramphenicol and streak purified on the same medium.

Lactococcus lactis strain MG1363 is transformed separately with the vector pSH71-ERY^r-TAL (example 22), yielding the strain FSLL-TAL; with pSH71-ERY^r-PAL-C4H (example 21), yielding the strain FSLL-PALC4H and with pSH71-CM^r-4CL-VST (example 23), yielding strain FSLL-4CLVST. In addition, *Lactococcus lactis* strain MG1363 is co-transformed with pSH71-ERY^r-TAL (example 22) and pSH71-CM^r-4CL-VST (example 23), and the transformed strain is named FSLL-TAL4CLVST. The same strain is also co-transformed with pSH71-ERY^r-PAL-C4H (example 21), and pSH71-CM^r-4CL-VST (example 23), resulting in the strain FSLL-PALC4H4CLVST.

Example 25

Fermentation with recombinant Lactococcus lactis strains in fermentors.

The recombinant yeast strains can be grown in fermenters operated as batch, fed-batch or chemostat cultures.

Batch and Fed-batch cultivations

The microorganism is grown in a baffled bioreactor with a working volume of 1.5 liters under anaerobic, aerobic or

microaerobic conditions. All cultures are incubated at 30°C, at 350 rpm. A constant pH of 6.6 is maintained by automatic addition of 10 M KOH. Cells are grown on lactose in defined MS10 medium supplemented with the following components to allow growth under aerobic conditions: $MnSO_4$ (1.25×10^{-5} g/l), thiamine (1 mg/l), and DL-6,8-thioctic acid (2.5 mg/l). The lactose concentration is, for example 50 g/l. The bioreactors are inoculated with cells from precultures grown at 30°C in shake flasks on the medium described above buffered with threefold-higher concentrations of K_2HPO_4 and KH_2PO_4. Anaerobic conditions are ensured by flushing the medium with N_2 (99.998% pure) prior to inoculation and by maintaining a constant flow of 50 ml/min of N_2 through the headspace of the bioreactor during cultivation. The bioreactors used for microaerobic and aerobic cultivation are equipped with polarographic oxygen sensors that are calibrated with air (DOT, 100%) and N_2 (DOT, 0%). Aerobic conditions are obtained by sparging the bioreactor with air at a rate of 1 vvm to ensure that the DOT is more than 80%. During microaerobic experiments the DOT is kept constant 5% by sparging the reactor with gas composed of a mixture of N_2 and atmospheric air, at a rate of 0.25 vvm.

Chemostat cultures

In chemostat cultures the cells can be grown in, for example, 1-L working-volume Applikon laboratory fermentors at 30 °C and 350 rpm. The dilution rate (*D*) can be set at different values, e.g. at 0.050 h^{-1}, 0.10 h^{-1}, 0.15 h^{-1}, or 0.20 h^{-1}. The pH is kept constant, e.g at 6.6, by automatic addition of 5 M KOH, using the growth medium described above, supplemented with antifoam (50 µl/l). The concentration of

lactose can be set at different values, e.g. is 3.0 g/l 6.0 g/l, 12.0 g/l, 15.0 g/l or 18.0 g/l. The bioreactor is inoculated to an initial biomass concentration of 1 mg /l and the feed pump is turned on at the end of the exponential growth phase.

An anaerobic steady state is obtained by introducing 50 ml/min of N_2 (99.998% pure) into the headspace of the bioreactor. Different anoxic steady states can obtained by sparging the reactor with 250 ml/min of gas composed of N_2 (99.998% pure) and atmospheric air at various ratios. The oxygen electrode is calibrated by sparging the bioreactor with air (100% DOT) and with N_2 (0% DOT).

For all conditions, the gas is sterile filtered before being introduced into the bioreactor. The off gas is led through a condenser cooled to lower than ~8°C and analyzed for its volumetric content of CO_2 and O_2 by means of an acoustic gas analyser.

Cultivations are considered to be in steady state after at least 5 residence times, and if the concentrations of biomass and fermentation end products remain unchanged (less than 5% relative deviation) over the last two residence times.

Example 26

Extraction and analyis of resveratrol in Lactococcus lactis

Extraction and analysis is performed using the methods as described in examples 14 and 15.

Example 27

Construction of a fungal vector for expression of PAL and C4H in species belonging to the genus Aspergillus.

The plasmid that is used in the following examples, is derived from pARp1 that contains the AMA1 initiating replication sequence from *Aspergillus nidulans*, which also sustains autonomous plasmid replication in *A. niger* and *A. oryzae* (Gems *et al.*, 1991). Moreover, the plasmid is a shuttle vector, containing the replication sequence of *Escherichia coli*, and the inherent difficult transformations in *Aspergillus niger* and *Aspergillus oryzae* can therefore overcome by using *Escherichia coli* as an intermediate host for the construction of recombinant plasmids. The plasmid contains one or more marker genes to allow the microorganism that harbour them to be selected from those which do not. The selection system can be either based upon dominant markers e.g. resistance against hygromycin B, phleomycin and bleomycin, or heterologous markers e.g amino acids and the *pyrG* gene. In addition the plasmid contains promoter- and terminator sequences that allow the expression of the recombinant genes. Suitable promoters are taken from genes of *Aspergillus nidulans e.g. alcA, glaA, amy, niaD, and gpdA*. Furthermore, the plasmid contains suitable unique restriction sites to facilitate the cloning of DNA fragments and subsequent identification of recombinants.

The plasmid used in the following examples contains the strong constitutive *gpdA*-promoter and auxotropic markers, all originating from *Aspergillus nidulans*; the plasmid containing the gene *methG* that is involved in methionine biosynthesis, is designated as pAMA1-MET; the plasmid containing the gene *hisA* that is involved in histidine biosynthesis, is designated as pAMA1-HIS.

The gene encoding PAL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-PAL-C4H (example 3), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the *gpdA* promoter from *Aspergillus nidulans*. The resulting plasmid, pAMA1-MET-PAL contains the gene encoding PAL under the control of the *gpdA* promoter from *Aspergillus nidulans*.

The gene encoding C4H, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-PAL-C4H (example 3) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-HIS vector to yield pAMA1-HIS-C4H. The *gpdA* promoter and the gene encoding C4H are reamplified as one fragment by PCR from the plasmid pAMA1-HIS-C4H using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pAMA1-MET-PAL. The resulting plasmid, pAMA1-MET-PAL-C4H, contains the genes encoding PAL and C4H that are each under the control of an individual *pgdA* promoter from *Aspergillus nidulans*. The sequence of the genes encoding PAL and C4H is verified by sequencing of two different clones of pAMA1-MET-PAL-C4H.

Example 28

Construction of a fungal vector for expression of TAL in species belonging to the genus Aspergillus.

The gene encoding for TAL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-URA-TAL (example 6) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector. The resulting plasmid, pAMA1-MET-TAL, contains the gene encoding for TAL under the control of the *gpdA* promoter from *Aspergillus nidulans*. The sequence of the gene encoding for TAL is verified by sequencing of two different clones of pAMA1-MET-TAL.

Example 29

Construction of a fungal vector for expression of 4CL and VST in species belonging to the genus Aspergillus.

The gene encoding 4CL, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-TRP-4CL-VST (example 5), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-HIS vector that contains the *gpdA* promoter from *Aspergillus nidulans*. The resulting plasmid, pAMA1-HIS-4CL contains the gene encoding 4CL under the control of the *gpdA* promoter from *Aspergillus nidulans*.

The gene encoding VST, isolated as described in example 1, is reamplified by PCR from the plasmid pESC-TRP-4CL-VST (example 5) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The

introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector to yield pAMA1-MET-VST. The *gpdA* promoter and the gene encoding VST are reamplified as one fragment by PCR from the plasmid pAMA1-MET-VST using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pAMA1-HIS-4CL. The resulting plasmid, pAMA1-HIS-4CL-VST, contains the genes encoding 4CL and VST that are each under the control of an individual *pgdA* promoter from *Aspergillus nidulans*. The sequence of the genes encoding 4CL and VST is verified by sequencing of two different clones of pAMA1-HIS-4CL-VST.

Example 30

Expression of the pathway to resveratrol in Aspergillus niger.

Aspergillus niger strains are transformed with the vectors described in examples 27, 28 and 29, separately or in combination. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook *et al.*, 1989). Transformants are selected on minimal medium lacking methionine and/or histidine.

A strain of *Aspergilus niger* that is auxotrophic for histidine and methionine, for instance, strain FGSC A919 (see http://www.fgsc.net), is transformed separately with the vector pAMA1-MET-TAL (example 28), yielding the strain FSAN-TAL; with pAMA1-MET-PAL-C4H (example 27), yielding the

strain FSAN-PALC4H and with pAMA1-HIS-4CL-VST (example 29), yielding strain FSAN-4CLVST. In addition, *Aspergillus niger* strain FGSC A919 is co-transformed with pAMA1-MET-TAL (example 28) and pAMA1-HIS-4CL-VST (example 29), and the transformed strain is named FSAN-TAL4CLVST. The same strain is also co-transformed with pAMA1-MET-PAL-C4H (example 27), and pAMA1-HIS-4CL-VST (example 29), resulting in the strain FSAN-PALC4H4CLVST.

Example 31

Expression of the pathway to resveratrol in Aspergillus oryzae.

A strain of *Aspergillus oryzae* that contains a native set of genes encoding for PAL, C4H and 4CL (Seshime *et al.*, 2005) and that is auxotrophic for methionine, is transformed with the vector pAMA1-MET-VST (example 29), yielding the strain FSAO-VST. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook *et al.*, 1989). Transformants are selected on minimal medium lacking methionine.

Example 32

Fermentation with recombinant strains of Aspergillus niger and Aspergillus oryzae in fermentors.

The recombinant yeast strains can be grown in fermenters operated as batch, fed-batch or chemostat cultures.

Batch and Fed-batch cultivations

The microorganism is grown in a baffled bioreactor with a working volume of 1.5 liters under aerobic conditions. All cultures are incubated at 30 °C, at 500 rpm. A constant pH of 6.0 is maintained by automatic addition of 10 M KOH, and aerobic conditions are obtained by sparging the bioreactor with air at a rate of 1 vvm to ensure that the DOT is more than 80%. Cells are grown on glucose in defined medium consisting of the following components to allow growth in batch cultivations: 7.3 g/l $(NH_4)_2SO_4$, 1.5 g/l KH_2PO_4, 1.0 g/l $MgSO_4.7H_2O$, 1.0 g/l NaCl, 0.1 g/l $CaCl_2.2H_2O$, 0.1 ml/l Sigma antifoam, 7.2 mg/l $ZnSO_4.7H_2O$, 1.3 mg/l $CuSO_4.5H_2O$, 0.3 mg/l $NiCl_2.6H_2O$, 3.5 mg/l $MnCl_2.4H_2O$ and 6.9 mg/l $FeSO_4.7H_2O$. The glucose concentration is, for example, 10- 20-, 30-, 40- or 50 g/l. To allow growth in fed-batch cultivations the medium is composed of: 7.3 g/l $(NH_4)_2SO_4$, 4.0 g/l KH_2PO_4, 1.9 g/l $MgSO_4.7H_2O$, 1.3 g/l NaCl, 0.10 g/l $CaCl_2.2H_2O$, 0.1 ml/l Sigma antifoam, 7.2 mg/l $ZnSO_4.7H_2O$, 1.3 mg/l $CuSO_4.5H_2O$, 0.3 mg/l $NiCl_2.6H_2O$, 3.5 mg/l $MnCl_2.4H_2O$ and 6.9 mg/l $FeSO_4.H_2O$ in the batch phase. The reactor is then fed with, for example, 285 g/kg glucose and 42 g/kg $(NH_4)_2SO_4$.

Free mycelium from a pre-batch is used for inoculating the batch- and fed-batch cultures. A spore concentration of 2.10^9 spores/l is used for inoculation of the pre-batch culture at pH 2.5. Spores are obtained by propagation of freeze-dried spores onto 29 g rice to which the following components are added: 6 ml 15 g/l sucrose, 2.3 g/l $(NH_4)_2SO_4$, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.50 g/l NaCl, 14.3 mg/l $ZnSO_4.7H_2O$, 2.5 mg/ $CuSO_4.5H_2O$, 0.50 mg/l $NiCl_2.6H_2O$, and 13.8 mg/l $FeSO_4.7H_2O$. The spores are propagated at 30 °C for 7-14 days to yield a black layer of spores on the rice grains and are harvested by adding 100 ml of 0.1% Tween 20 in sterile water. For all conditions, the gas is sterile filtered before being introduced into the bioreactor. The off gas is

led through a condenser cooled to lower than −8°C and analyzed for its volumetric content of CO_2 and O_2 by means of an acoustic gas analyser.

Chemostat cultures

In chemostat cultures the cells can be grown in, for example, 1.5-L working-volume Biostat B laboratory fermentors at 30 °C and 500 rpm. A constant pH of 6.0 is maintained by automatic addition of 10 M KOH, and aerobic conditions are obtained by sparging the bioreactor with air at a rate of 1 vvm to ensure that the DOT is more than 80%. The dilution rate (*D*) can be set at different values, e.g. at 0.050 h^{-1}, 0.10 h^{-1}, 0.15 h^{-1}, or 0.20 h^{-1}. The pH is kept constant, e.g at 6.6, by automatic addition of 10 M KOH, using a minimal growth medium with the following components: 2.5 g/l $(NH_4)_2SO_4$, 0.75 g/l KH_2PO_4, 1.0 g/l $MgSO_4.7H_2O$, 1.0 g/l NaCl, 0.1 g/l $CaCl_2.2H_2O$, 0.1 ml/l Sigma antifoam, 7.2 mg/l $ZnSO_4.7H_2O$, 1.3 mg/l $CuSO_4.5H_2O$, 0.3 mg/l $NiCl_2.6H_2O$, 3.5 mg/l $MnCl_2.4H_2O$ and 6.9 mg/l $FeSO_4.7H_2O$. The concentration of glucose can be set at different values, e.g. is 3.0 g/l 6.0 g/l, 12.0 g/l, 15.0 g/l or 18.0 g/l. The bioreactor is inoculated with free mycelium from a pre-batch culture as described above, and the feed pump is turned on at the end of the exponential growth phase.

For all conditions, the gas is sterile filtered before being introduced into the bioreactor. The off gas is led through a condenser cooled to lower than −8°C and analyzed for its volumetric content of CO_2 and O_2 by means of an acoustic gas analyser.

Cultivations are considered to be in steady state after at least 5 residence times, and if the concentrations of

biomass glucose and composition of the off-gas remain unchanged (less than 5% relative deviation) over the last two residence times.

Example 33

Extraction and analyis of resveratrol in Aspergillus niger and Aspergillus oryzae

Extraction and analysis is performed using the methods as described in examples 14 and 15.

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The following is a summary of the nucleotide and amino acid sequences appearing herein:

SEQ ID NO: 1 is a nucleotide sequence from *Arabidopsis thaliana* encoding a phenylalanine ammonia lyase (PAL2).
SEQ ID NO: 2 is the amino acid sequence encoded by SEQ ID NO: 1.
SEQ ID NO: 3 is a nucleotide sequence from *Arabidopsis thaliana* encoding a cinnamate 4-hydroxylase (C4H).
SEQ ID NO: 4 is the amino acid sequence encoded by SEQ ID NO: 3.
SEQ ID NO: 5 is a nucleotide sequence from *Arabidopsis thaliana* encoding a 4-coumarate:CoenzymeA ligase (4CL1).
SEQ ID NO: 6 is the amino acid sequence encoded by SEQ ID NO: 5.
SEQ ID NO: 7 is a nucleotide sequence from *Rheum tataricum* encoding a resveratrol synthase (VST).
SEQ ID NO: 8 is the amino acid sequence encoded by SEQ ID NO: 7.
SEQ ID NO: 9 is a nucleotide sequence from *Rheum tataricum* encoding a resveratrol synthase (VST), which is codon-optimized for expression in *S. cerevisiae*.
SEQ ID NO: 10 is the amino acid sequence encoded by SEQ ID NO: 9.

SEQ ID NO: 11 is a nucleotide sequence from *Rhodobacter capsulatus* encoding a tyrosine ammonia lyase (TAL).

SEQ ID NO: 12 is the amino acid sequence encoded by SEQ ID NO: 11.

SEQ ID NO: 13 is a nucleotide sequence from *Rhodobacter capsulatus* encoding a tyrosine ammonia lyase (TAL), which is codon-optimized for expression in *S. cerevisiae*.

SEQ ID NO: 14 is the amino acid sequence encoded by SEQ ID NO: 13.

SEQ ID NO: 15 is a nucleotide sequence from *S. cerevisiae* encoding a NADPH:cytochrome P450 reductase (CPR1).

SEQ ID NO: 16 is the amino acid sequence encoded by SEQ ID NO: 15.

SEQ ID NO: 17 is a nucleotide sequence from *Arabidopsis thalianus* encoding a NADPH:cytochrome P450 reductase (AR2).

SEQ ID NO: 18 is the amino acid sequence encoded by SEQ ID NO: 17.

SEQ ID NOs 19-32 are primer sequences appearing in Table 1, Example 1.

SEQ ID NOs 33-34 are primer sequences appearing in Example 16.

SEQ ID NOs 35-38 are primer sequences appearing in Example 17

SEQUENCE LISTING

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Ile Val Leu Ala Ile Ala Lys Ser Ser Glu Thr Glu Lys Tyr Asp Leu
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Ser Ser Ile Arg Val Val Lys Ser Gly Ala Ala Pro Leu Gly Lys Glu
                325                 330                 335

Leu Glu Asp Ala Val Asn Ala Lys Phe Pro Asn Ala Lys Leu Gly Gln
            340                 345                 350

Gly Tyr Gly Met Thr Glu Ala Gly Pro Val Leu Ala Met Ser Leu Gly
        355                 360                 365

Phe Ala Lys Glu Pro Phe Pro Val Lys Ser Gly Ala Cys Gly Thr Val
    370                 375                 380

Val Arg Asn Ala Glu Met Lys Ile Val Asp Pro Asp Thr Gly Asp Ser
385                 390                 395                 400

Leu Ser Arg Asn Gln Pro Gly Glu Ile Cys Ile Arg Gly His Gln Ile
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405 410 415

Met Lys Gly Tyr Leu Asn Asn Pro Ala Ala Thr Ala Glu Thr Ile Asp
420 425 430

Lys Asp Gly Trp Leu His Thr Gly Asp Ile Gly Leu Ile Asp Asp Asp
435 440 445

Asp Glu Leu Phe Ile Val Asp Arg Leu Lys Glu Leu Ile Lys Tyr Lys
450 455 460

Gly Phe Gln Val Ala Pro Ala Glu Leu Glu Ala Leu Leu Ile Gly His
465 470 475 480

Pro Asp Ile Thr Asp Val Ala Val Val Ala Met Lys Glu Glu Ala Ala
485 490 495

Gly Glu Val Pro Val Ala Phe Val Val Lys Ser Lys Asp Ser Glu Leu
500 505 510

Ser Glu Asp Asp Val Lys Gln Phe Val Ser Lys Gln Val Val Phe Tyr
515 520 525

Lys Arg Ile Asn Lys Val Phe Phe Thr Glu Ser Ile Pro Lys Ala Pro
530 535 540

Ser Gly Lys Ile Leu Arg Lys Asp Leu Arg Ala Lys Leu Ala Asn Gly
545 550 555 560

Leu

<210> 7
<211> 1176
<212> DNA
<213> Rheum tataricum

<400> 7
atggcaccgg aggagtccag gcatgctgaa actgcagtta acagagccgc caccgtcctg 60

gccatcggca ctgccaaccc gccaaactgc tactatcaag cggactttcc tgacttctac 120

ttccgtgcca ccaacagcga ccacctcacg cacctcaagc aaaaatttaa gcgcatttgt 180

gagaaatcga tgattgaaaa acgttatctc catttgacgg aagaaattct caaggagaat 240

ccaaatattg cttccttcga ggcgccatca ttggatgtaa gacataacat tcaagtgaaa 300

gaagtggtgc tgctcggaaa agaggcagct ttgaaggcca tcaatgagtg gggccaaccc 360

aagtcaaaga tcacgcgcct cattgtgtgt tgtattgccg gcgttgacat gcccggcgca 420

gactatcaac tcactaaact ccttggctta caactttctg ttaagcgatt tatgttttac 480

cacctaggat gctatgccgg tggcaccgtc cttcgccttg cgaaggacat agcagaaaac 540

aacaaggaag ctcgtgttct catcgttcgc tctgagatga cgccaatctg tttccgtggg 600

ccatccgaaa cccacataga ctccatggta gggcaagcaa tatttggtga cggtgctgcg 660

gctgttatag ttggtgcaaa tcccgaccta tccatcgaaa ggccgatttt cgagttgatt 720

tctacatccc aaactatcat acctgaatcc gatggtgcga ttgagggaca tttgcttgaa 780

gttggactca gtttccaact ctaccagact gttccctcat taatctctaa ttgtatcgaa 840

acttgtcttt caaaggcttt cacacctctt aacattagtg attggaactc actattctgg 900

attgcacacc ctggtggccg tgctatcctt gacgatatcg aggctactgt tggtctcaag 960

aaggagaaac ttaaggcaac aagacaagtt ttgaacgact atgggaacat gtcaagtgct 1020

tgcgtatttt tcatcatgga tgagatgagg aagaagtcgc tcgcaaacgg tcaagtaacc 1080

actggagaag gactcaagtg gggtgttctt tttgggttcg ggccaggtgt tactgtggaa 1140

actgtggttc taagcagtgt gccgctaatt acctga 1176

<210> 8
<211> 391
<212> PRT
<213> Rheum tataricum

<400> 8

Met Ala Pro Glu Glu Ser Arg His Ala Glu Thr Ala Val Asn Arg Ala
1 5 10 15

Ala Thr Val Leu Ala Ile Gly Thr Ala Asn Pro Pro Asn Cys Tyr Tyr
20 25 30

Gln Ala Asp Phe Pro Asp Phe Tyr Phe Arg Ala Thr Asn Ser Asp His
35 40 45

Leu Thr His Leu Lys Gln Lys Phe Lys Arg Ile Cys Glu Lys Ser Met
50 55 60

Ile Glu Lys Arg Tyr Leu His Leu Thr Glu Glu Ile Leu Lys Glu Asn
65 70 75 80

Pro Asn Ile Ala Ser Phe Glu Ala Pro Ser Leu Asp Val Arg His Asn

85 90 95

Ile Gln Val Lys Glu Val Val Leu Leu Gly Lys Glu Ala Ala Leu Lys
100 105 110

Ala Ile Asn Glu Trp Gly Gln Pro Lys Ser Lys Ile Thr Arg Leu Ile
115 120 125

Val Cys Cys Ile Ala Gly Val Asp Met Pro Gly Ala Asp Tyr Gln Leu
130 135 140

Thr Lys Leu Leu Gly Leu Gln Leu Ser Val Lys Arg Phe Met Phe Tyr
145 150 155 160

His Leu Gly Cys Tyr Ala Gly Gly Thr Val Leu Arg Leu Ala Lys Asp
165 170 175

Ile Ala Glu Asn Asn Lys Glu Ala Arg Val Leu Ile Val Arg Ser Glu
180 185 190

Met Thr Pro Ile Cys Phe Arg Gly Pro Ser Glu Thr His Ile Asp Ser
195 200 205

Met Val Gly Gln Ala Ile Phe Gly Asp Gly Ala Ala Ala Val Ile Val
210 215 220

Gly Ala Asn Pro Asp Leu Ser Ile Glu Arg Pro Ile Phe Glu Leu Ile
225 230 235 240

Ser Thr Ser Gln Thr Ile Ile Pro Glu Ser Asp Gly Ala Ile Glu Gly
245 250 255

His Leu Leu Glu Val Gly Leu Ser Phe Gln Leu Tyr Gln Thr Val Pro
260 265 270

Ser Leu Ile Ser Asn Cys Ile Glu Thr Cys Leu Ser Lys Ala Phe Thr
275 280 285

Pro Leu Asn Ile Ser Asp Trp Asn Ser Leu Phe Trp Ile Ala His Pro
290 295 300

Gly Gly Arg Ala Ile Leu Asp Asp Ile Glu Ala Thr Val Gly Leu Lys
305 310 315 320

Lys Glu Lys Leu Lys Ala Thr Arg Gln Val Leu Asn Asp Tyr Gly Asn
325 330 335

Met Ser Ser Ala Cys Val Phe Phe Ile Met Asp Glu Met Arg Lys Lys
340 345 350

Ser Leu Ala Asn Gly Gln Val Thr Thr Gly Glu Gly Leu Lys Trp Gly
355 360 365

Val Leu Phe Gly Phe Gly Pro Gly Val Thr Val Glu Thr Val Val Leu
370 375 380

Ser Ser Val Pro Leu Ile Thr
385 390

<210> 9
<211> 1176
<212> DNA
<213> Rheum tataricum

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gctattggta cggccaatcc acccaattgt tactatcaag ctgactttcc tgatttttat 120

ttcagagcca caaatagcga tcatttgact catcttaagc aaaaatttaa aaggatatgc 180

gagaagtcca tgattgaaaa gagatacttg caccttaccg aagagatctt aaaagaaaac 240

ccaaatatag cttcttttga agctccctcc ttagatgtac gtcacaacat tcaagtcaag 300

gaggtggttt tacttggtaa ggaagccgca ttgaaagcta taaacgaatg gggacagcct 360

aaaagtaaga taaccagatt gatcgtatgt tgcatagctg gcgttgacat gcctggtgca 420

gattatcaac taacaaaatt gctgggtcta caattatccg taaaaaggtt tatgttctac 480

catttaggct gttacgctgg tggcacagtt ttaagactgg ctaaggatat agcagaaaat 540

aacaaggagg ctagagtctt aatagtgcgt agtgaaatga ctcctatttg ctttagaggt 600

ccatcagaaa cacatatcga cagcatggta ggtcaggcaa ttttcggtga tggtgctgca 660

gccgtaattg tgggagctaa tcctgattta agtatcgaaa gacctatttt tgaacttatt 720

tctacttcgc aaaccattat ccccgaatca gatggtgcaa ttgaaggcca tttattggag 780

gttggtttgt cctttcaatt gtatcagaca gtgccatctt taatttcaaa ctgtatagaa 840

acctgtctaa gtaaagcatt tacaccatta aacatttctg actggaattc tttgttctgg 900

attgctcatc caggtggaag agccatctta gatgacatcg aagctactgt gggactgaaa 960

aaggaaaaac taaaagctac tagacaagtt ttaaatgact acggtaatat gtcatctgct 1020

tgtgtatttt tcattatgga tgagatgaga aaaaagtcac ttgcaaatgg ccaggtcacg 1080

acaggtgagg gtctaaaatg gggagtccta ttcggattcg gcccaggtgt cactgttgaa 1140

accgttgtcc tgtcttcggt tccattgatc acttaa 1176

<210> 10
<211> 391
<212> PRT
<213> Rheum tataricum

<400> 10

Met Ala Pro Glu Glu Ser Arg His Ala Glu Thr Ala Val Asn Arg Ala
1 5 10 15

Ala Thr Val Leu Ala Ile Gly Thr Ala Asn Pro Pro Asn Cys Tyr Tyr
20 25 30

Gln Ala Asp Phe Pro Asp Phe Tyr Phe Arg Ala Thr Asn Ser Asp His
35 40 45

Leu Thr His Leu Lys Gln Lys Phe Lys Arg Ile Cys Glu Lys Ser Met
50 55 60

Ile Glu Lys Arg Tyr Leu His Leu Thr Glu Glu Ile Leu Lys Glu Asn
65 70 75 80

Pro Asn Ile Ala Ser Phe Glu Ala Pro Ser Leu Asp Val Arg His Asn
85 90 95

Ile Gln Val Lys Glu Val Val Leu Leu Gly Lys Glu Ala Ala Leu Lys
100 105 110

Ala Ile Asn Glu Trp Gly Gln Pro Lys Ser Lys Ile Thr Arg Leu Ile
115 120 125

Val Cys Cys Ile Ala Gly Val Asp Met Pro Gly Ala Asp Tyr Gln Leu
130 135 140

Thr Lys Leu Leu Gly Leu Gln Leu Ser Val Lys Arg Phe Met Phe Tyr
145 150 155 160

His Leu Gly Cys Tyr Ala Gly Gly Thr Val Leu Arg Leu Ala Lys Asp
165 170 175

Ile Ala Glu Asn Asn Lys Glu Ala Arg Val Leu Ile Val Arg Ser Glu
180 185 190

Met Thr Pro Ile Cys Phe Arg Gly Pro Ser Glu Thr His Ile Asp Ser
195 200 205

Met Val Gly Gln Ala Ile Phe Gly Asp Gly Ala Ala Ala Val Ile Val
210 215 220

Gly Ala Asn Pro Asp Leu Ser Ile Glu Arg Pro Ile Phe Glu Leu Ile
225 230 235 240

Ser Thr Ser Gln Thr Ile Ile Pro Glu Ser Asp Gly Ala Ile Glu Gly
245 250 255

His Leu Leu Glu Val Gly Leu Ser Phe Gln Leu Tyr Gln Thr Val Pro
260 265 270

Ser Leu Ile Ser Asn Cys Ile Glu Thr Cys Leu Ser Lys Ala Phe Thr
275 280 285

Pro Leu Asn Ile Ser Asp Trp Asn Ser Leu Phe Trp Ile Ala His Pro
290 295 300

Gly Gly Arg Ala Ile Leu Asp Asp Ile Glu Ala Thr Val Gly Leu Lys
305 310 315 320

Lys Glu Lys Leu Lys Ala Thr Arg Gln Val Leu Asn Asp Tyr Gly Asn
325 330 335

Met Ser Ser Ala Cys Val Phe Phe Ile Met Asp Glu Met Arg Lys Lys
340 345 350

Ser Leu Ala Asn Gly Gln Val Thr Thr Gly Glu Gly Leu Lys Trp Gly
355 360 365

Val Leu Phe Gly Phe Gly Pro Gly Val Thr Val Glu Thr Val Val Leu
370 375 380

Ser Ser Val Pro Leu Ile Thr
385 390

<210> 11
<211> 1596
<212> DNA

<213> Rhodobacter capsulatus

<400> 11

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gtccaatgcg aggccatcgc gacacatcgc agccggattt cggtgacccc cgcgctgcgc 120

gagcgctgcg cgcgggccca tgcccggctt gagcacgcca tcgccgagca gcgccacatt 180

tacggcatca ccaccggctt cggcccgctg gcgaaccgtc tgatcggggc cgatcagggg 240

gcggagctgc agcagaacct gatctatcat ctggccaccg gcgtcgggcc gaaactgagc 300

tgggccgagg cgcgggcgtt gatgctggcg cggctcaact cgatcctgca aggcgcgtcg 360

ggggcctcgc cggagacgat cgaccggatc gttgcggtgc tcaatgcggg gtttgccccc 420

gaggttccgg cgcagggaac ggtgggcgcc tcgggcgatc tgaccccgct tgcgcatatg 480

gtgctggcgc tgcagggacg ggggcggatg atcgacccct cgggccgcgt gcaggaggcc 540

ggggcggtga tggatcggct ctgcggcggt ccgctgacgc tggcggcccg tgacgggctg 600

gcgctggtga atggcacctc ggcgatgacc gcgattgcgg ccctgaccgg ggtcgaggcg 660

gcgcgggcga tcgacgccgc gcttcggcac agcgcggtcc tgatggaggt cttgtccggt 720

catgccgaag cctggcatcc ggctttcgca gagctgcgcc cgcatccggg gcagctgcgg 780

gcgaccgagc ggctggcgca ggcgctggat ggggcggggc gggtctgtcg gaccctgacc 840

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tacagtctgc gcgtggtgcc gcaactggtc ggcgcggtct gggacacgct ggactggcac 960

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tgcgcggtgc ccgccctgca cggcggcaat ttcatgggcg tgcatgtcgc ccttgcctcc 1080

gatgcgctga acgcggcgct ggtgacgctg gcgggcctgg tcgagcgtca gatcgcccgg 1140

ctgaccgacg aaaagctgaa caagggcctg cccgccttcc tgcacggggg gcaggcgggg 1200

ctgcaatcgg gcttcatggg ggcgcaggtc acggcgacgg cgcttctggc ggaaatgcgg 1260

gcgaatgcca cgccggtttc ggtgcagtcg ctgtcgacca atggcgccaa tcaggatgtg 1320

gtctcgatgg gaacgattgc cgcgcggagg gcgcgggcgc agctgctgcc cctgtcgcag 1380

atccaggcga tcctggcgct tgcccttgcc caggcgatgg atctgcttga cgaccccgag 1440

gggcaggccg gatggtcgct tacggcgcgg gatctgcggg accggatccg ggcggtctcg 1500

cccgggcttc gcgccgacag accgcttgcc gggcatatcg aagcggtggc acagggtctg 1560

cgtcatccct ccgccgccgc cgatcccccg gcatga 1596

<210> 12

<211> 531
<212> PRT
<213> Rhodobacter capsulatus

<400> 12

Met Thr Leu Gln Ser Gln Thr Ala Lys Asp Cys Leu Ala Leu Asp Gly
1 5 10 15

Ala Leu Thr Leu Val Gln Cys Glu Ala Ile Ala Thr His Arg Ser Arg
20 25 30

Ile Ser Val Thr Pro Ala Leu Arg Glu Arg Cys Ala Arg Ala His Ala
35 40 45

Arg Leu Glu His Ala Ile Ala Glu Gln Arg His Ile Tyr Gly Ile Thr
50 55 60

Thr Gly Phe Gly Pro Leu Ala Asn Arg Leu Ile Gly Ala Asp Gln Gly
65 70 75 80

Ala Glu Leu Gln Gln Asn Leu Ile Tyr His Leu Ala Thr Gly Val Gly
85 90 95

Pro Lys Leu Ser Trp Ala Glu Ala Arg Ala Leu Met Leu Ala Arg Leu
100 105 110

Asn Ser Ile Leu Gln Gly Ala Ser Gly Ala Ser Pro Glu Thr Ile Asp
115 120 125

Arg Ile Val Ala Val Leu Asn Ala Gly Phe Ala Pro Glu Val Pro Ala
130 135 140

Gln Gly Thr Val Gly Ala Ser Gly Asp Leu Thr Pro Leu Ala His Met
145 150 155 160

Val Leu Ala Leu Gln Gly Arg Gly Arg Met Ile Asp Pro Ser Gly Arg
165 170 175

Val Gln Glu Ala Gly Ala Val Met Asp Arg Leu Cys Gly Gly Pro Leu
180 185 190

Thr Leu Ala Ala Arg Asp Gly Leu Ala Leu Val Asn Gly Thr Ser Ala
195 200 205

Met Thr Ala Ile Ala Ala Leu Thr Gly Val Glu Ala Ala Arg Ala Ile

210 215 220

Asp Ala Ala Leu Arg His Ser Ala Val Leu Met Glu Val Leu Ser Gly
225 230 235 240

His Ala Glu Ala Trp His Pro Ala Phe Ala Glu Leu Arg Pro His Pro
245 250 255

Gly Gln Leu Arg Ala Thr Glu Arg Leu Ala Gln Ala Leu Asp Gly Ala
260 265 270

Gly Arg Val Cys Arg Thr Leu Thr Ala Ala Arg Arg Leu Thr Ala Ala
275 280 285

Asp Leu Arg Pro Glu Asp His Pro Ala Gln Asp Ala Tyr Ser Leu Arg
290 295 300

Val Val Pro Gln Leu Val Gly Ala Val Trp Asp Thr Leu Asp Trp His
305 310 315 320

Asp Arg Val Val Thr Cys Glu Leu Asn Ser Val Thr Asp Asn Pro Ile
325 330 335

Phe Pro Glu Gly Cys Ala Val Pro Ala Leu His Gly Gly Asn Phe Met
340 345 350

Gly Val His Val Ala Leu Ala Ser Asp Ala Leu Asn Ala Ala Leu Val
355 360 365

Thr Leu Ala Gly Leu Val Glu Arg Gln Ile Ala Arg Leu Thr Asp Glu
370 375 380

Lys Leu Asn Lys Gly Leu Pro Ala Phe Leu His Gly Gly Gln Ala Gly
385 390 395 400

Leu Gln Ser Gly Phe Met Gly Ala Gln Val Thr Ala Thr Ala Leu Leu
405 410 415

Ala Glu Met Arg Ala Asn Ala Thr Pro Val Ser Val Gln Ser Leu Ser
420 425 430

Thr Asn Gly Ala Asn Gln Asp Val Val Ser Met Gly Thr Ile Ala Ala
435 440 445

Arg Arg Ala Arg Ala Gln Leu Leu Pro Leu Ser Gln Ile Gln Ala Ile
450 455 460

Leu Ala Leu Ala Leu Ala Gln Ala Met Asp Leu Leu Asp Asp Pro Glu
465 470 475 480

Gly Gln Ala Gly Trp Ser Leu Thr Ala Arg Asp Leu Arg Asp Arg Ile
485 490 495

Arg Ala Val Ser Pro Gly Leu Arg Ala Asp Arg Pro Leu Ala Gly His
500 505 510

Ile Glu Ala Val Ala Gln Gly Leu Arg His Pro Ser Ala Ala Ala Asp
515 520 525

Pro Pro Ala
530

<210> 13
<211> 1596
<212> DNA
<213> Rhodobacter capsulatus

<400> 13
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gttcaatgcg aagcgatagc aacccataga agtagaatct ctgtaacacc agccctacgt 120

gagagatgtg ctagagcaca tgctaggtta gaacatgcaa tagccgaaca gcgacacata 180

tatgggataa cgacaggctt cgggccactt gctaacaggc tgatcggagc agaccagggt 240

gctgaattac aacagaacct tatctaccat ttggcaaccg gagttggccc caaattatca 300

tgggccgaag ccagagcttt aatgctcgct cgtttgaata gtatactaca aggtgcttct 360

ggtgctagcc ctgaaacaat tgataggatc gttgcagtct taaatgccgg atttgccccg 420

gaagtcccag cccaaggaac cgttggtgct tcgggtgact taactccgtt agcacacatg 480

gtattagcat tgcaaggcag aggtcgtatg attgatcctt cagggagagt tcaagaagcc 540

ggcgctgtca tggataggtt gtgtggaggc cctttaacat tggctgccag agatggcctc 600

gccttagtaa atggtacatc tgccatgaca gctattgccg cattgaccgg tgtggaggct 660

gcaagagcga ttgatgcagc gcttagacat tccgcagtct tgatggaggt cctgtcaggg 720

catgctgagg cttggcaccc tgcctttgcg gaattgcgtc cgcatccagg acaattacgc 780

gccactgaga ggttagctca agcattggac ggcgcaggta gagtctgccg gactcttaca 840

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gctaacgcga ctcccgtgtc cgttcaaagc ctcagcacca atggtgcaaa tcaagacgtg 1320

gtaagtatgg gtacgattgc cgcgagacga gcaagagctc aacttttacc tctgtctcaa 1380

atccaagcga ttttggcact ggctcttgca caagccatgg atctcctaga cgatcctgaa 1440

ggacaagccg gttggtcctt aacggcaaga gatttaagag accgtatacg ggctgtcagt 1500

ccagggttgc gcgcagatag accactagcg ggtcatattg aagctgtggc tcaaggtcta 1560

agacacccct cggcagctgc cgatccacct gcttaa 1596

<210> 14
<211> 531
<212> PRT
<213> Rhodobacter capsulatus

<400> 14

Met Thr Leu Gln Ser Gln Thr Ala Lys Asp Cys Leu Ala Leu Asp Gly
1 5 10 15

Ala Leu Thr Leu Val Gln Cys Glu Ala Ile Ala Thr His Arg Ser Arg
20 25 30

Ile Ser Val Thr Pro Ala Leu Arg Glu Arg Cys Ala Arg Ala His Ala
35 40 45

Arg Leu Glu His Ala Ile Ala Glu Gln Arg His Ile Tyr Gly Ile Thr
50 55 60

Thr Gly Phe Gly Pro Leu Ala Asn Arg Leu Ile Gly Ala Asp Gln Gly
65 70 75 80

Ala Glu Leu Gln Gln Asn Leu Ile Tyr His Leu Ala Thr Gly Val Gly
85 90 95

Pro Lys Leu Ser Trp Ala Glu Ala Arg Ala Leu Met Leu Ala Arg Leu
100 105 110

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Asn Ser Ile Leu Gln Gly Ala Ser Gly Ala Ser Pro Glu Thr Ile Asp
        115                 120                 125

Arg Ile Val Ala Val Leu Asn Ala Gly Phe Ala Pro Glu Val Pro Ala
    130                 135                 140

Gln Gly Thr Val Gly Ala Ser Gly Asp Leu Thr Pro Leu Ala His Met
145                 150                 155                 160

Val Leu Ala Leu Gln Gly Arg Gly Arg Met Ile Asp Pro Ser Gly Arg
                165                 170                 175

Val Gln Glu Ala Gly Ala Val Met Asp Arg Leu Cys Gly Gly Pro Leu
            180                 185                 190

Thr Leu Ala Ala Arg Asp Gly Leu Ala Leu Val Asn Gly Thr Ser Ala
        195                 200                 205

Met Thr Ala Ile Ala Ala Leu Thr Gly Val Glu Ala Ala Arg Ala Ile
    210                 215                 220

Asp Ala Ala Leu Arg His Ser Ala Val Leu Met Glu Val Leu Ser Gly
225                 230                 235                 240

His Ala Glu Ala Trp His Pro Ala Phe Ala Glu Leu Arg Pro His Pro
                245                 250                 255

Gly Gln Leu Arg Ala Thr Glu Arg Leu Ala Gln Ala Leu Asp Gly Ala
            260                 265                 270

Gly Arg Val Cys Arg Thr Leu Thr Ala Ala Arg Arg Leu Thr Ala Ala
        275                 280                 285

Asp Leu Arg Pro Glu Asp His Pro Ala Gln Asp Ala Tyr Ser Leu Arg
    290                 295                 300

Val Val Pro Gln Leu Val Gly Ala Val Trp Asp Thr Leu Asp Trp His
305                 310                 315                 320

Asp Arg Val Val Thr Cys Glu Leu Asn Ser Val Thr Asp Asn Pro Ile
                325                 330                 335

Phe Pro Glu Gly Cys Ala Val Pro Ala Leu His Gly Gly Asn Phe Met
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340 345 350

Gly Val His Val Ala Leu Ala Ser Asp Ala Leu Asn Ala Ala Leu Val
355 360 365

Thr Leu Ala Gly Leu Val Glu Arg Gln Ile Ala Arg Leu Thr Asp Glu
370 375 380

Lys Leu Asn Lys Gly Leu Pro Ala Phe Leu His Gly Gly Gln Ala Gly
385 390 395 400

Leu Gln Ser Gly Phe Met Gly Ala Gln Val Thr Ala Thr Ala Leu Leu
405 410 415

Ala Glu Met Arg Ala Asn Ala Thr Pro Val Ser Val Gln Ser Leu Ser
420 425 430

Thr Asn Gly Ala Asn Gln Asp Val Val Ser Met Gly Thr Ile Ala Ala
435 440 445

Arg Arg Ala Arg Ala Gln Leu Leu Pro Leu Ser Gln Ile Gln Ala Ile
450 455 460

Leu Ala Leu Ala Leu Ala Gln Ala Met Asp Leu Leu Asp Asp Pro Glu
465 470 475 480

Gly Gln Ala Gly Trp Ser Leu Thr Ala Arg Asp Leu Arg Asp Arg Ile
485 490 495

Arg Ala Val Ser Pro Gly Leu Arg Ala Asp Arg Pro Leu Ala Gly His
500 505 510

Ile Glu Ala Val Ala Gln Gly Leu Arg His Pro Ser Ala Ala Ala Asp
515 520 525

Pro Pro Ala
530

<210> 15
<211> 2076
<212> DNA
<213> Saccharomyces cerevisiae

<400> 15
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acagctgtca gctcgggcaa cagagacatt gctcaggtgg tgaccgaaaa caacaagaac 180
tacttggtgt tgtatgcgtc gcagactggg actgccgagg attacgccaa aaagttttcc 240
aaggagctgg tggccaagtt caacctaaac gtgatgtgcg cagatgttga gaactacgac 300
tttgagtcgc taaacgatgt gcccgtcata gtctcgattt ttatctctac atatggtgaa 360
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gcactatcga acctgaggta taatatgttt ggtctgggaa attctactta tgaattcttt 480
aatggtgccg ccaagaaggc cgagaagcat ctctccgccg cgggcgctat cagactaggc 540
aagctcggtg aagctgatga tggtgcagga actacagacg aagattacat ggcctggaag 600
gactccatcc tggaggtttt gaaagacgaa ctgcatttgg acgaacagga agccaagttc 660
acctctcaat tccagtacac tgtgttgaac gaaatcactg actccatgtc gcttggtgaa 720
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tcttccaatg accgtaattg catccactct gaatttgact tgtccggctc taacatcaag 900
tactccactg gtgaccatct tgctgtttgg ccttccaacc cattggaaaa ggtcgaacag 960
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gctgacgtca aggaaaaatt gactctgctt tcgaaagaca aggaccaatt cgccgtcgag 1200
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Claims

1. A micro-organism having an operative metabolic pathway comprising at least one enzyme activity, said pathway producing 4-coumaric acid and producing resveratrol therefrom, or an oligomeric or glycosidically-bound derivative thereof.

2. A micro-organism as claimed in claim 1, wherein said resveratrol is produced in a reaction catalysed by an enzyme in which endogenous malonyl-CoA is a substrate.

3. A micro-organism as claimed in claim 1 or claim 2, wherein said resveratrol is produced from 4-coumaroyl-CoA.

4. A micro-organism as claimed in claim 3, wherein said resveratrol is produced from 4-coumaroyl-CoA by a resveratrol synthase.

5. A micro-organism as claimed in claim 4, wherein said resveratrol synthase is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

6. A micro-organism as claimed in claim 5, wherein said resveratrol synthase is resveratrol synthase (EC 2.3.1.95) from a plant belonging to the genus of *Arachis*, a plant belonging to the genus of *Rheum*, or a plant belonging to the genus of *Vitus* or any one of the genera *Pinus*, *Picea*, *Lilium*, *Eucalyptus*, *Parthenocissus*, *Cissus*, *Calochortus*,

Polygonum, *Gnetum*, *Artocarpus*, *Nothofagus*, *Phoenix*, *Festuca*, *Carex*, *Veratrum*, *Bauhinia* or *Pterolobium*.

7. A micro-organism as claimed in any preceding claim, wherein said 4-coumaric acid is produced from *trans*-cinnamic acid.

8. A micro-organism as claimed in claim 7, wherein said 4-coumaric acid is produced from *trans*-cinnamic acid by an enzyme in a reaction catalysed by said enzyme in which oxygen is a substrate, NADH or NADPH is a cofactor and NAD or NADP is a product.

9. A micro-organism as claimed in claim 8, wherein said 4-coumaric acid is produced from *trans*-cinnamic acid by a cinnamate 4-hydroxylase.

10. A micro-organism as claimed in claim 9, wherein said cinnamate 4-hydroxylase is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

11. A micro-organism as claimed in claim 10, wherein said cinnamate 4-hydroxylase is cinnamate 4-hydroxylase (EC 1.14.13.11) from a plant belonging to the genus of *Arabidopsis*, a plant belonging to the genus of *Citrus*, a plant belonging to the genus of *Phaseolus*, a plant belonging to the genus of *Pinus*, a plant belonging to the genus of *Populus*, a plant belonging to the genus of *Solanum*, a plant belonging to the genus of *Vitus*, a plant belonging to the genus of *Zea*, or any one of the genera *Ammi*, *Avicennia*, *Camellia*, *Camptotheca*, *Catharanthus*, *Glycine*, *Helianthus*, *Lotus*, *Mesembryanthemum*,

Physcomitrella, *Ruta*, *Saccharum*, and *Vigna*, *from a* filamentous fungus belonging to the genus *Aspergillus*.

12. A micro-organism as claimed in any one of claims 1 to 6, wherein said 4-coumaric acid is produced from tyrosine in a reaction catalysed by an enzyme in which ammonia is produced.

13. A micro-organism as claimed in any one of claims 1 to 4, wherein said 4-coumaric acid is produced from *tyrosine* by a L-phenylalanine ammonia lyase or a tyrosine ammonia lyase.

14. A micro-organism as claimed in claim 13, wherein said 4-coumaric acid is produced by tyrosine ammonia lyase (EC 4.3.1.5) from yeast or bacteria.

15. A micro-organism as claimed in claim 14, wherein said tyrosine ammonia lyase is from the yeast *Rhodotorula rubra* or from the bacterium *Rhodobacter capsulatus*.

16. A micro-organism as claimed in any one of claims 12 to 15, wherein said tyrosine ammonia lyase is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

17. A micro-organism as claimed in any one of claims 9 to 11, wherein said *trans*-cinnamic acid is produced from L-phenylalanine in a reaction catalysed by an enzyme in which ammonia is produced.

18. A micro-organism as claimed in claim 17, wherein said *trans*-cinnamic acid is formed from L-phenylalanine by a L-phenylalanine ammonia lyase.

19. A micro-organism as claimed in claim 18, wherein said *trans*-cinnamic acid is formed from L-phenylalanine by L-phenylalanine ammonia lyase (EC 4.3.1.5) from a plant belonging to the genus of *Arabidopsis*, a plant belonging to the genus of *Brassica*, a plant belonging to the genus of *Citrus*, a plant belonging to the genus of *Phaseolus*, a plant belonging to the genus of *Pinus*, a plant belonging to the genus of *Populus*, a plant belonging to the genus of *Solanum*, a plant belonging to the genus of *Prunus*, a plant belonging to the genus of *Vitus*, a plant belonging to the genus of *Zea*, or a plant belonging to any one of the genera *Agastache*, *Ananas*, *Asparagus*, *Bromheadia*, *Bambusa*, *Beta*, *Betula*, *Cucumis*, *Camellia*, *Capsicum*, *Cassia*, *Catharanthus*, *Cicer*, *Citrullus*, *Coffea*, *Cucurbita*, *Cynodon*, *Daucus*, *Dendrobium*, *Dianthus*, *Digitalis*, *Dioscorea*, *Eucalyptus*, *Gallus*, *Ginkgo*, *Glycine*, *Hordeum*, *Helianthus*, *Ipomoea*, *Lactuca*, *Lithospermum*, *Lotus*, *Lycopersicon*, *Medicago*, *Malus*, *Manihot*, *Medicago*, *Mesembryanthemum*, *Nicotiana*, *Olea*, *Oryza*, *Pisum*, *Persea*, *Petroselinum*, *Phalaenopsis*, *Phyllostachys*, *Physcomitrella*, *Picea*, *Pyrus*, *Quercus*, *Raphanus*, *Rehmannia*, *Rubus*, *Sorghum*, *Sphenostylis*, *Stellaria*, *Stylosanthes*, *Triticum*, *Trifolium*, *Triticum*, *Vaccinium*, *Vigna*, or *Zinnia*, or from a filamentous fungus belonging to the genus *Aspergillus*.

20. A micro-organism as claimed in any one of claims 18 or claim 19, wherein said phenylalanine ammonia lyase is expressed in said micro-organism from nucleic acid coding for said enzyme which is not native to the micro-organism.

21. A micro-organism as claimed in any preceding claim, wherein 4-coumaroyl-CoA is formed in a reaction catalysed by an enzyme in which ATP and CoA are substrates and ADP is a product.

22. A micro-organism as claimed in any preceding claim, wherein 4-coumaroyl-CoA is formed in a reaction catalysed by a 4-coumarate-CoA ligase.

23. A micro-organism as claimed in claim 22, wherein said 4-coumarate-CoA ligase is 4-coumarate-CoA ligase (EC 6.2.1.12) from a plant belonging to the genus of *Abies*, a plant belonging to the genus of *Arabidopsis*, a plant belonging to the genus of *Brassica*, a plant belonging to the genus of *Citrus*, a plant belonging to the genus of *Larix*, a plant belonging to the genus of *Phaseolus*, a plant belonging to the genus of *Pinus*, a plant belonging to the genus of *Populus*, a plant belonging to the genus of *Solanum*, a plant belonging to the genus of *Vitus*, a plant belonging to the genus of *Zea*, or a plant belonging to any one of the genera *Agastache*, *Amorpha*, *Cathaya*, *Cedrus*, *Crocus*, *Festuca*, *Glycine*, *Juglans*, *Keteleeria*, *Lithospermum*, *Lolium*, *Lotus*, *Lycopersicon*, *Malus*, *Medicago*, *Mesembryanthemum*, *Nicotiana*, *Nothotsuga*, *Oryza*, *Pelargonium*, *Petroselinum*, *Physcomitrella*, *Picea*, *Prunus*, *Pseudolarix*, *Pseudotsuga*, *Rosa*, *Rubus*, *Ryza*, *Saccharum*, *Suaeda*, *Thellungiella*, *Triticum*, or *Tsuga*, *from a* filamentous fungus belonging to the genus *Aspergillus*, a filamentous fungus belonging to the genus *Neurospora*, a fungus belonging to the genus *Yarrowia*, a fungus belonging to the genus of *Mycosphaerella*, from a bacterium belonging to the genus of *Mycobacterium*, a bacterium belonging to

the genus of *Neisseria*, a bacterium belonging to the genus of *Streptomyces*, a bacterium belonging to the genus of *Rhodobacter*, or from a nematode belonging to the genus *Ancylostoma*, a nematode belonging to the genus *Caenorhabditis*, a nematode belonging to the genus *Haemonchus*, a nematode belonging to the genus *Lumbricus*, a nematode belonging to the genus *Meilodogyne*, a nematode belonging to the genus *Strongyloidus*, or a nematode belonging to the genus *Pristionchus*.

24. A micro-organism as claimed in any preceding claim, wherein at least one copy of at least one genetic sequence encoding a respective enzyme in said metabolic pathway has been recombinantly introduced into said micro-organism.

25. A micro-organism as claimed in claim 24, wherein a native NADPH:cytochrome P450 reductase (CPR) has been overexpressed in said micro-organism.

26. A micro-organism as claimed in claim 24, wherein a NADPH:cytochrome P450 reductase (CPR) has been recombinantly introduced into said micro-organism.

27. A micro-organism as claimed in claim 26, wherein said NADPH:cytochrome P450 reductase is NADPH:cytochrome P450 reductase (EC 1.6.2.4) from a plant belonging to the genus of *Arabidopsis*, e.g. *A. thaliana*, a plant belonging to the genus of *Citrus*, e.g. *C. sinensis*, *C. x paradisi*, a plant belonging to the genus of *Phaseolus*, e.g. *P. vulgaris*, a plant belonging to the genus of *Pinus*, e.g. *P. taeda*, a plant belonging to the genus of *Populus*, e.g. *P. deltoides*, *P. tremuloides*, *P. trichocarpa*, a plant belonging to the genus of *Solanum*, e.g. *S. tuberosum*, a

plant belonging to the genus of *Vitus*, e.g. *Vitus vinifera*, a plant belonging to the genus of *Zea*, e.g. *Z. mays*, or other plant genera e.g. *Ammi*, *Avicennia*, *Camellia*, *Camptotheca*, *Catharanthus*, *Glycine*, *Helianthus*, *Lotus*, *Mesembryanthemum*, *Physcomitrella*, *Ruta*, *Saccharum*, *Vigna*.

28. A micro-organism as claimed in any preceding claim, wherein at least one copy of a genetic sequence encoding a tyrosine ammonia lyase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

29. A micro-organism as claimed in any preceding claim wherein at least one copy of a genetic sequence encoding a phenylalanine ammonia lyase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

30. A micro-organism as claimed in any preceding claim wherein at least one copy of a genetic sequence encoding cinnamate 4-hydroxylase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

31. A micro-organism as claimed in any preceding claim wherein at least one copy of a genetic sequence encoding a 4-coumarate-CoA ligase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

32. A micro-organism as claimed in any preceding claim wherein at least one copy of a genetic sequence encoding a

resveratrol synthase is operatively linked to an expression signal not natively associated with said genetic sequence in said organism.

33. A micro-organism as claimed in any preceding claim, which is a fungus.

34. A micro-organism as claimed in claim 33, which is a filamentous fungi.

35. A micro-organism as claimed in claim 34, which is a micro-organism belonging to the genus *Aspergillus*.

36. A micro-organism as claimed in claim 35, which is a strain of *Aspergillus niger or A. oryzae*.

37. A micro-organism as claimed in 33, which is a yeast.

38. A micro-organism as claimed in claim 37, which is a micro-organism belonging to the genus *Saccharomyces, Klyuveromyces, Candida, Pichia, Debaromyces, Hansenula, Yarrowia, Zygosaccharomyces or Schizosaccharomyces.*

39. A micro-organism as claimed in claim 38, which is a strain of *Saccharomyces cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum , Klyuveromyces lactis K. marxianus var. marxianus, K. thermotolerans, Candida utilis C. tropicalis, Pichia stipidis, P. pastoris, P. sorbitophila, Debaromyces hansenii, Hansenula polymorpha, Yarrowia lipolytica, Zygosaccharomyces rouxii* or *Schizosaccharomyces pombe*.

40. A micro-organism as claimed in any one of claim 1 to 32, which is a bacterium.

41. A micro-organism as claimed in claim 40, which is a micro-organism belonging to the genus *Escherichia or Lactococcus*.

42. A micro-organism as claimed in claim 41, which is a strain of *Escherichia coli or Lactococcus lactis*.

43. A micro-organism as claimed in claim 1, containing one or more copies of an heterologous DNA sequence encoding phenylalanine ammonia lyase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding cinnamate-4-hydroxylase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding 4-coumarate CoA-ligase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding resveratrol synthase operatively associated with an expression signal.

44. A micro-organism as claimed in claim 1, lacking cinnamate-4-hydroxylase activity, and containing one or more copies of a heterologous DNA sequence encoding tyrosine ammonia lyase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding 4-coumarate CoA-ligase operatively associated with an expression signal, and containing one or more copies of an heterologous DNA sequence encoding resveratrol synthase operatively associated with an expression signal.

45. A method for producing resveratrol or an oligomeric or glycosidically-bound derivative thereof comprising contacting a non-plant cell having an appropriate resveratrol producing metabolic pathway with a carbon substrate in the substantial absence of an external source of 4-coumaric acid.

46. A method as claimed in claim 45, wherein said non-plant cell is selected from the group consisting of fungi and bacteria.

47. A method as claimed in claim 46, where said non-plant cell is a fungus selected from the group of yeast.

48. A method as claimed in claim 47, where said yeast is selected from the species *Saccharomyes*.

49. A method as claimed in any one of claims 45 to 48, wherein said non-plant cell is of a micro-organism as claimed in any one of claims 1 to 44.

50. A method as claimed in any one of claim 45 to 49, wherein said carbon substrate is selected from the group of fermentable carbon substrates consisting of monosaccharides, oligosaccharides and polysaccharides.

51. A method as claimed in claim 50, wherein said fermentable carbon substrate is glucose, fructose, galactose, xylose, arabinose, mannose, sucrose, lactose, erythrose, threose, ribose.

52. A method as claimed in any one of claim 45 to 49, wherein said carbon substrate is selected from the group of non-fermentable carbon substrate.

53. A method as claimed in claim 52, wherein said non-fermentable carbon substrate is ethanol, acetate, glycerol, lactate.

54. A method as claimed in claim 52, wherein said non-fermentable carbon substrate is selected from the group consisting of amino acids.

55. A method as claimed in claim 54, wherein said non-fermentable carbon substrate is selected from the group consisting of phenylalanine and tyrosine.

56. A method for producing resveratrol or an oligomeric or glycosidically-bound derivative thereof through heterologous expression of nucleotide sequences encoding phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate-CoA ligase and resveratrol synthase.

57. A method for producing resveratrol or an oligomeric or glycosidically-bound derivative thereof through heterologous expression of nucleotide sequences encoding tyrosine ammonia lyase, 4-coumarate-CoA ligase and resveratrol synthase.

58. A method as claimed in any one of claims 45 to 57, further including using said produced resveratrol or an oligomeric or glycosidically-bound derivative thereof as a nutraceutical in a dairy product or a beverage.

59. A method as claimed in claim 58, wherein said resveratrol or an oligomeric or glycosidically-bound derivative thereof is used as a nutraceutical in beer.

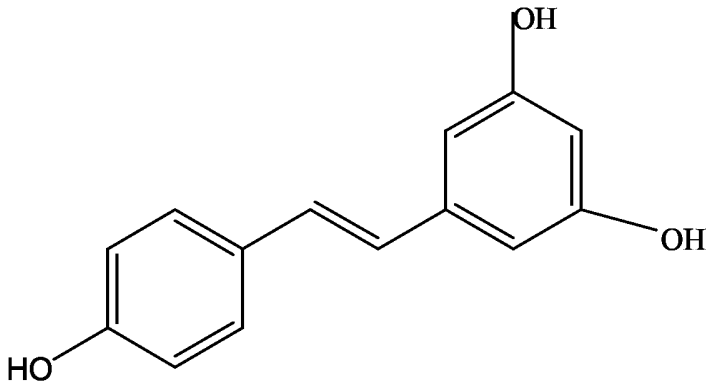


Figure 1

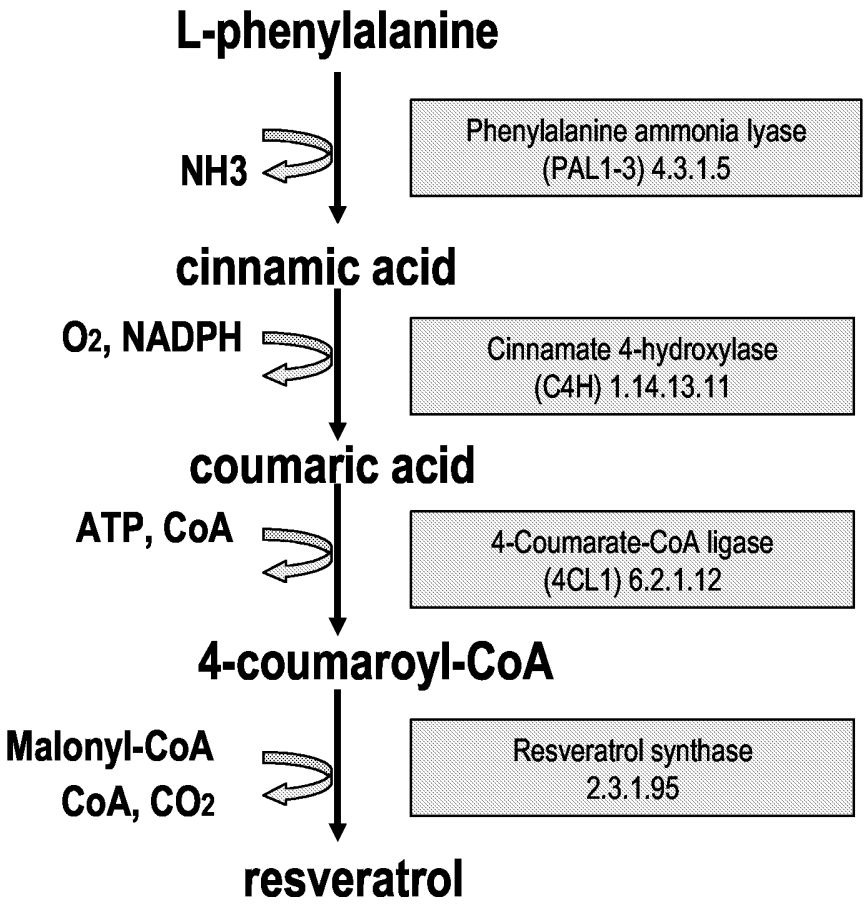

L-phenylalanine
NH3
Phenylalanine ammonia lyase
(PAL1-3) 4.3.1.5
cinnamic acid
O2, NADPH
Cinnamate 4-hydroxylase
(C4H) 1.14.13.11
coumaric acid
ATP, CoA
4-Coumarate-CoA ligase
(4CL1) 6.2.1.12
4-coumaroyl-CoA
Malonyl-CoA
CoA, CO2
Resveratrol synthase
2.3.1.95
resveratrol

Figure 2

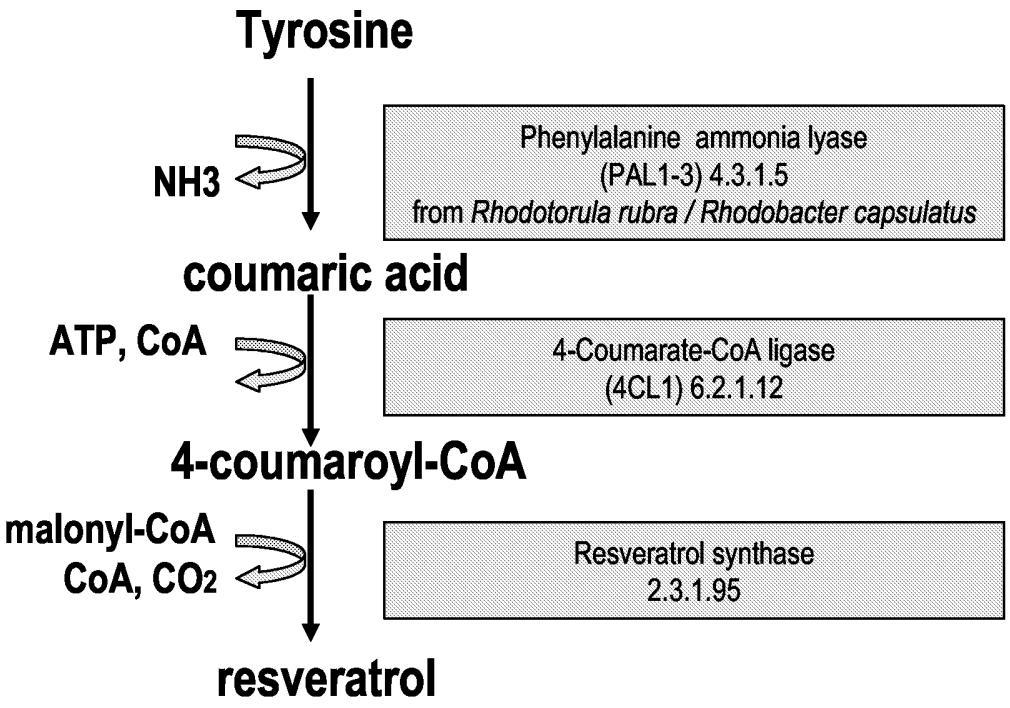
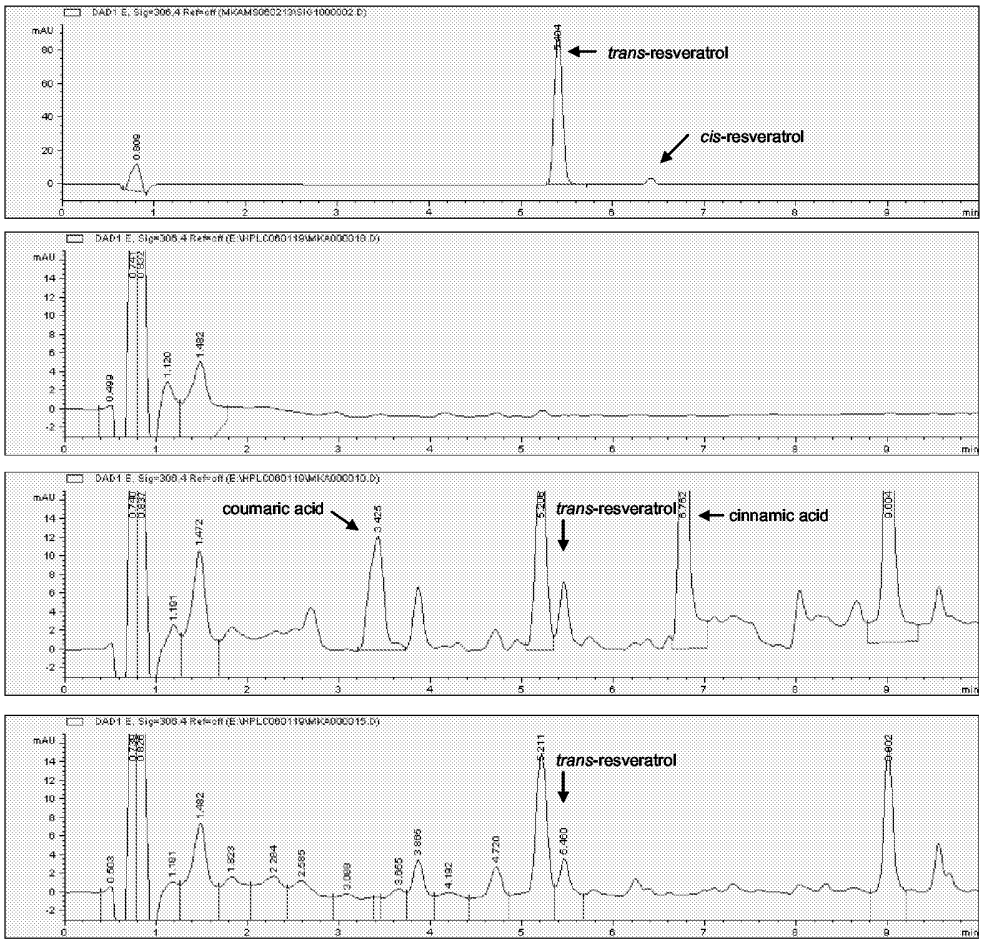

Tyrosine
NH3
Phenylalanine ammonia lyase
(PAL1-3) 4.3.1.5
from Rhodotorula rubra / Rhodobacter capsulatus
coumaric acid
ATP, CoA
4-Coumarate-CoA ligase
(4CL1) 6.2.1.12
4-coumaroyl-CoA
malonyl-CoA
CoA, CO2
Resveratrol synthase
2.3.1.95
resveratrol

Figure 3



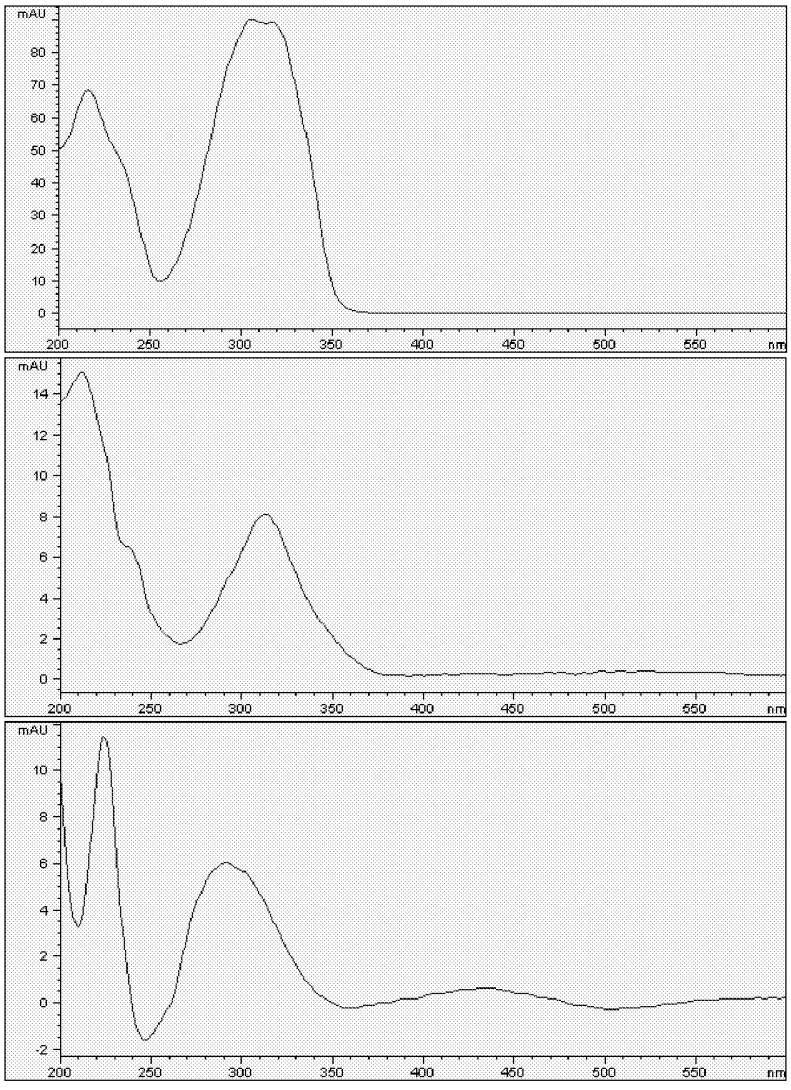
resveratrol standard
cis- and *trans*-form
60 nanogram total

S. cerevisiae strain:
FSSC-control
(empty vectors)

S. cerevisiae strain:
FSSC-PALC4H4CLVST
(PAL-pathway)

S. cerevisiae strain:
FSSC-TAL4CLVST
(TAL-pathway)

Figure 4

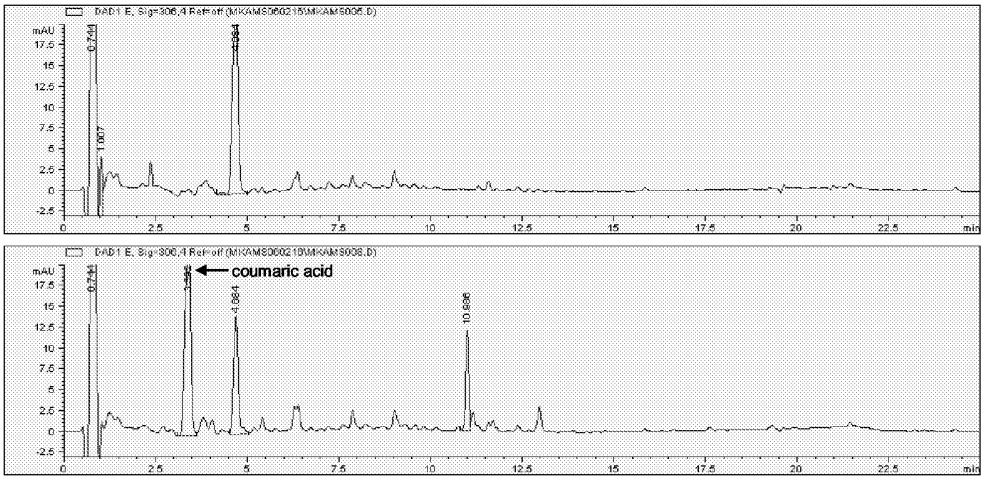


uv-spectrum of pure *trans*-resveratrol 60 nanogram total

uv-spectrum of *trans*-resveratrol in extract of *S. cerevisiae* strain FSSC-PALC4H4CLVST (PAL-pathway)

uv-spectrum of *trans*-resveratrol in extract of *S. cerevisiae* strain FSSC-TAL4CLVST (TAL-pathway)

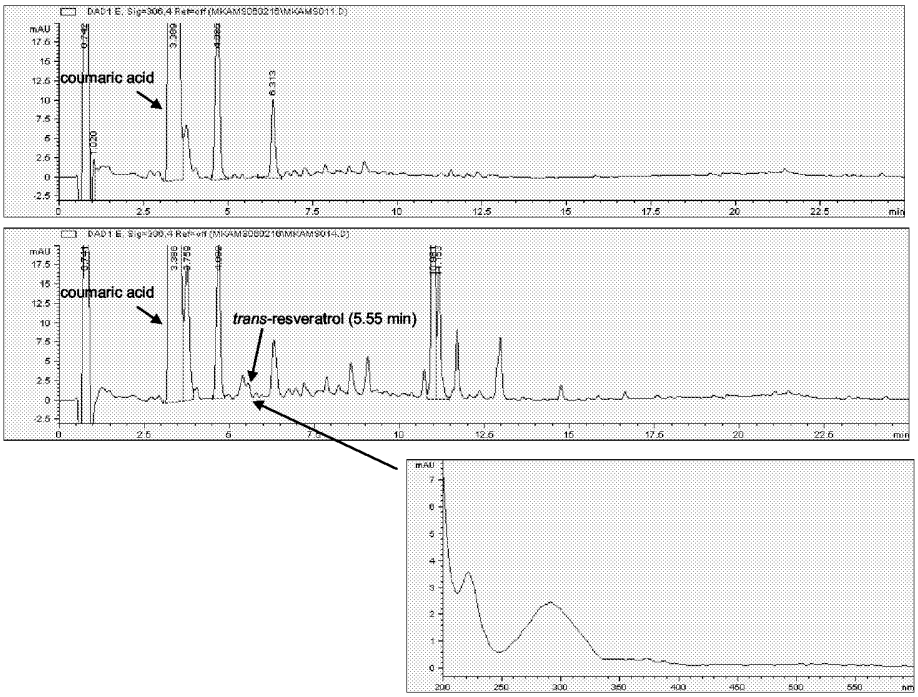
Figure 5



E. coli strain:
FSEC-control
(empty vectors)

E. coli strain:
FSEC-TAL4CLVST
(TAL-pathway)

Figure 6



E. coli strain:
FSEC-control
(empty vectors)
+
20 mg/l coumaric acid

E. coli strain:
FSEC-TAL4CLVST
(TAL-pathway)
+
20 mg/l coumaric acid

uv-spectrum of *trans*-resveratrol
in extract of *E. coli* strain
FSEC-TAL4CLVST
(TAL-pathway)

Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/060154

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/00 C12N9/02 C12N9/10 C12N9/88 C12P7/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAMAPPITO S ET AL: "Aromatic and pyrone polyketides synthesized by a stilbene synthase from Rheum tataricum" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 62, no. 3, February 2003 (2003-02), pages 313-323, XP004412246 ISSN: 0031-9422 cited in the application abstract ----- -/--	45,46

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2006	20/06/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Huse, I

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/060154

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BECKER JOHN V W ET AL: "Metabolic engineering of Saccharomyces cerevisiae for the synthesis of the wine-related antioxidant resveratrol." FEMS YEAST RESEARCH. OCT 2003, vol. 4, no. 1, October 2003 (2003-10), pages 79-85, XP002347057 ISSN: 1567-1356 cited in the application the whole document	1-59
Y	WATTS KEVIN T ET AL: "Exploring recombinant flavonoid biosynthesis in metabolically engineered Escherichia coli." CHEMBIOCHEM : A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY. 2 APR 2004, vol. 5, no. 4, 2 April 2004 (2004-04-02), pages 500-507, XP002347112 ISSN: 1439-4227 cited in the application abstract	1-59
Y	HWANG E I ET AL: "Production of plant-specific flavanones by Escherichia coli containing an artificial gene cluster" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, WASHINGTON,DC, US, vol. 69, no. 5, May 2003 (2003-05), pages 2699-2706, XP002977268 ISSN: 0099-2240 cited in the application abstract the whole document	1-59
A	RO DAE-KYUN ET AL: "Reconstitution of the entry point of plant phenylpropanoid metabolism in yeast (Saccharomyces cerevisiae): implications for control of metabolic flux into the phenylpropanoid pathway." THE JOURNAL OF BIOLOGICAL CHEMISTRY. 23 JAN 2004, vol. 279, no. 4, 23 January 2004 (2004-01-23), pages 2600-2607, XP002347058 ISSN: 0021-9258 cited in the application abstract page 2602, right-hand column - page 2603, left-hand column	

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/060154

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/10407 A (E.I. DUPONT DE NEMOURS AND COMPANY) 7 February 2002 (2002-02-07) abstract page 13, line 12 - line 36	
A	JEANDET PHILIPPE ET AL: "Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism." JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY. 8 MAY 2002, vol. 50, no. 10, 8 May 2002 (2002-05-08), pages 2731-2741, XP002347113 ISSN: 0021-8561 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/060154

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0210407 A	07-02-2002	EP 1303622 A1	23-04-2003
		JP 2004530402 T	07-10-2004